# Development Towards a Portable Instrument for the Determination of Pesticide Residue in Water

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## STATEMENT OF AUTHENTICITY

The following thesis; '*Development towards a portable instrument for the determination of pesticide residue in water*' is based on the work completed solely by the author and duly referenced where external assistance or knowledge was acquired. The work presented in this thesis is the work of the author and has not been submitted for the award of any other degree or diploma in any university.

The work described and presented in this research project was carried out in the School of Applied Sciences, RMIT, Melbourne, Australia between March 2004 and March 2011.

Signature Date

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*"Success starts with a dream or a thought; but that's only the first step. Writing it down and doing specific things to bring it about is the path to fulfilling the dream……"*

- **Author Unknown.**

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# LITERATURE CONTRIBUTIONS

***Journal Publications***

- Beale, D.J., Porter, N.A, & Roddick, F.A. (2009). '*A fast screening method for the presence of atrazine and other triazines in water using flow injection with chemiluminescent detection'.* **Talanta**, **78**(2): p. 342-347.
- Beale, D.J., Kaserzon, S.L., Porter, N.A., Roddick, F.A., & Carpenter, P. (2010). '*Detection of s-triazine pesticides in natural waters by modified large-volume direct injection HPLC'.* **Talanta**, **82**(2): p. 668-674*.*
- Beale, D.J., Porter, N.A., & Roddick, F.A. (2010). '*A semi-automated in-line SPE FICA for the determination of atrazine and atrazine metabolites*'. **Manuscript in preparation**.
- Beale, D.J., Porter, N.A., & Roddick, F.A. (2010). '*A multi-analyte in-line solid phase extraction flow injection chemiluminescent method for the determination of pesticide residue in water'.* **Manuscript in preparation**.

***Poster Presentations***

- Ratanachaithong M., Beale D.J., Porter N.A., & Roddick, F.A. (2005). '*Comparison of two chemiluminescent reagents for the determination of dicrotophos pesticide in water'.* Presented at 13th International Conference on Flow Injection Analysis, Nevada, USA.
- Beale D.J., Porter N.A., & Roddick, F.A. (2004). '*Development of a portable instrument for the determination of pesticide residue in aqueous samples'.* Presented at 14th RACI Research and Development Conference, University of Melbourne, Victoria.

***Conference Proceedings***

- Beale D., Porter N., & Roddick F. (2005). '*Atrazine detection by flow injection chemiluminescence'.* Presented at the 9th Environmental Research Event, University of Tasmainia, Hobart Campus, Tasmania.
- Beale D., Porter N., & Roddick F. (2006). *'Flow injection chemiluminescence analysis (FICA) of pesticides in natural waters'*. Presented at the 6th CRC for Water Quality and Treatment Postgraduate Conference, Melbourne, Victoria

- Ratanachaithong M., Beale D., Porter N., & Roddick F. (2005). '*Pesticide Detection in Water, An Illuminating Technique'*. Presented at CRC WQT Summer Research Seminars 2005, University of Adelaide – Mawson Lakes, South Australia.
- Enting N., Beale D., Porter N., & Considine R. (2007). '*Risk Analysis within a Melbourne Drinking Water Catchment'.* Presented at CRC WQT Summer Research Seminars 2007, Melbourne, Victoria.

# EXECUTIVE SUMMARY

## *Pesticide contamination*

Pesticide contamination has been a widely publicised topic over the past 30 years and will continue to be a heated discussion point in the future. As community awareness increases on the observed and potential impacts of pesticide use, more exposure and scrutiny is being applied to government agencies that control and regulate their use and the commercial entities that rely on them. Already we are seeing large scale pesticide contamination and community outrage within Australia.

## *Pesticide monitoring*

The monitoring and analysis of pesticides within the potable water sector is an expensive and daunting task for most water companies. The number of possible contaminants that can enter into the water supply is very large (both chemical and biological), and in areas of intense agriculture concentrations can be significantly higher (*e.g.* mg $L^{-1}$). Monitoring for all possible contaminates is both financially not feasible and physically impossible within current resource levels under which water companies operate.

Given the level of scrutiny the water sector operates under (namely water quality, water availability and pricing); a robust technique is needed for the determination of pesticide residues in source waters and water within the distribution system that is both cost effective and reliable. This research project is aimed at developing such a technique that can help water authorities meet this challenge, utilising flow injection analysis (FIA). As such, this thesis presents the application of FIA with chemiluminescence detection (FICA) for the determination of atrazine, simazine, hexazinone, monocrotophos, and dicrotophos in natural waters.

## *Pesticide determination by flow injection chemiluminesce analysis*

The FICA method presented utilises chemically oxidized chemiluminescent reagents, tris(2,2'-bipyridyl)ruthenium(III) and luminol, which have been successfully applied for the determination of compounds comprising an aliphatic amine moiety and organophosphates, respectively. A multivariate and univariate optimisation method was applied. The optimised tris(2,2'-bipyridyl)ruthenium(III) experimental conditions were: sample and carrier flow rates of 4.6 mL $min^{-1}$, sample at pH 9 buffered with 50 mM borax, and a reagent concentration of 1 mM tris(2,2'-bipyridyl)ruthenium(III) in 20 mM $H_2SO_4$ (pH 1). The developed optimised luminol experimental conditions for monocrotophos and dicrotophos were determined to

be: sample and carrier flow rates of 3.0 mL $min^{-1}$, sample at pH 9 buffered with 50 mM borax, and a reagent concentration of 2.75 mM luminol reagent in 0.1 M NaOH.

Once the operating conditions were defined, a series of experiments were carried out in order to further enhance the capabilities of the instrument and reduce the limits of detection to below Australian Drinking Water Guidelines (ADWG). The experiments comprised: analysis of pesticide residues in MilliQ water; analysis of pesticide residues in natural waters; analysis of pesticide residues with in-line solid phase extraction (SPE); and, analysis of multiple pesticides residues with in-line SPE and monolithic separation.

### *Atrazine analysis in MilliQ water and natural waters*

The analysis of pesticide residues in MilliQ water is presented where atrazine was detected using tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence with a limit of detection of 1.3 ± 0.1 µg $L^{-1}$. Validation of the method was performed by direct injection HPLC, with no significant difference observed between the methods ($R^2$ = 0.9906, t-test (6) = 0.39 (p two tailed = 0.71) and -0.74 (p two tailed = 0.48) for 0.5 and 10 µg $L^{-1}$ atrazine respectively). The HPLC method was further developed by incorporating a monolithic column which significantly decreased the analysis time. Analysis of natural waters comprising various concentrations of natural organic matter (represented as dissolved organic matter (DOM): 3.1 – 11.7 mg $L^{-1}$) by the modified HPLC had no significant effect on the resolution or separation capacity for atrazine, simazine and hexazinone. Monocrotophos and dicrotophos were deemed unsuitable for this analysis; both analytes co-eluted with the DOM peak.

While the application of FICA was successful when analysing atrazine in clean samples, when applied to natural waters the presence of DOM caused a significant positive chemiluminescent response. The functional groups responsible for the interference were identified by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) as amines and hydroxyl groups present in the natural water. In addition, the effect of various cations and anions was investigated at levels common in natural waters.

The interference from DOM was removed by SPE. As a result, the detection limit for atrazine in natural water samples was reduced to 14 ± 2 ng $L^{-1}$ with strong correlation with the HPLC method ($R^2$ = 0.9906, t-test (6) = 0.39 (p two tailed = 0.71) and -0.74 (p two tailed = 0.48) for 0.5 and 10 µg $L^{-1}$ atrazine respectively).

### *Comparison of reagents*

As a side study, the evaluation of luminol and tris(2'2-bipridyl)ruthenium(III) chemiluminescence was evaluated for the determination of monocrotophos and dicrotophos. It was successfully applied to the detection of dicrotophos (LOD 18.1 µg L-1) and monocrotophos (LOD 7.1 µg L-1) in MilliQ water and natural water samples containing DOM. Chemiluminescence generated using luminol was found to be better than with tris(2'2-bipridyl)ruthenium(III) for the selected organophosphates because of its greater sensitivity and freedom from interference. While the detection limit was above the current health trigger value set in the ADWG, it could be further reduced using online extraction and pre concentration.

### *Interference study*

Aside from the interference caused by DOM, a series of cations and anions were evaluated. It was observed that $Fe^{3+}$ and $Fe^{2+}$ (at concentrations above ADWG) caused interference. It was also shown that similar compounds, such as the atrazine metabolites and other triazine pesticides, produced a chemiluminescent signal with tris(2,2-bipyridyl)ruthenium(III).

Three dimensional excitation emission matrix (3DEEM) fluorescence spectroscopy was applied to natural water samples to investigate the interaction between DOM and the selected pesticides. It was observed that all three classes of pesticides inhibited the fluorescence intensity at the humic-like (A) and (C) fluorophore, and in the presence of Fe, Al and Cu were observed to shift or increase the fluorophore intensity. The fluorphore associated with each pesticide observed in neat water samples (MilliQ) was not present in natural waters containing DOM. As such, it is concluded a pesticide-natural organic matter complex was being formed.

### *Pesticide determination with in-line solid phase extraction*

The incorporation of an in-line extraction column enables the rapid detection of pesticide residues that had previously proven to be difficult due to interfering species. A variety of extraction resins were evaluated, namely: MIEX© (used to remove DOM) and, C18 and Nexus© (used to trap target analytes). It was found that Nexus© increased the capacity for larger extractions to be undertaken due to the multi-layer absorption capacity of the resin. This allowed the rapid analysis of smaller volumes (*i.e.,* 100 ml) to be carried out without an off-line extraction. The analysis of atrazine, hexazinone, and simazine by in-line SPE (with

Nexus©) was successfully applied with method detection limits of 14, 48 and 32 ng $L^{-1}$, respectively. No positive interferences were observed.

### *Multiple pesticide flow injection chemiluminescence analysis*

The benefits of a monolithic column (*e.g.* low pressure chromatographic separation) were merged with the advantages of the in-line SPE in order to create a hybrid FICA system analogous to a low pressure HPLC system. The incorporation of a monolithic column enabled atrazine, simazine and hexazinone to be detected simultaneously with chromatographic differentiation, with method detection limits of 27, 39 and 60 ng $L^{-1}$, respectively.

### *Conclusions*

Overall, the FICA system described in this thesis will be very useful as a quick, sensitive screening method for atrazine, simazine, hexazinone and selected metabolites in natural waters. The methods developed during the course of this project should be considered by water utilities for inclusion in their ongoing pesticide monitoring programs.

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# CHAPTER ONE: INTRODUCTION

This thesis presents an extensive review of the available literature (*i.e.,* journals, '*grey*' literature and government reports) to provide a basis for the experimental work proposed and completed for the fulfilment of a doctorate of philosophy by research. The experimental work presented herein describes the application of flow injection analysis (FIA) to a series of water samples in order to identify and detect pesticide residues below drinking water guidelines. From the literature, atrazine, simazine, hexazinone, monocrotophos and dicrotophos were selected as pesticides for investigation based on their history and likelihood to contaminant water ways. In addition, these pesticides comprise similar functional groups (*e.g.* aliphatic amines). Aliphatic amines are known to chemiluminescence with tris(2,2'-bipyridyl)ruthenium(III); this is the detection mechanism used in the FIA instrument.

In order to achieve this goal, a series of FIA experiments were conducted, namely: analysis of water samples without pre concentration, analysis of water samples with off-line pre concentration, analysis of water samples with in-line pre concentration, and lastly, analysis of water samples with pre concentration with in-line separation and differentiation.

As such, this thesis is structured in following way: first, a review of the available literature is presented (including history of the selected pesticides, common methods of detection and the proposed FIA method). Second, the method details and a list of consumables used are presented. This is followed by a series of chapters outlining the experiments undertaken in order fulfil the project objectives. Lastly, a summary of the research findings is presented along with a pathway of future research needs.

## 1.1. Focus of the Investigation

The monitoring and analysis of pesticide residues within the potable water sector is an expensive and daunting task for most water companies. The number of possible contaminants (both chemical and biological) that can enter into the water supply is very large, and in areas of intense agriculture concentrations can be significantly higher than background concentrations (*e.g.* in the magnitude of mg $L^{-1}$); however, the difficulty isn't in

monitoring waters contaminated with high concentration of pesticide residue, it is when these compounds are found at trace levels (*e.g.* in the magnitude of μg $L^{-1}$ down to pg $L^{-1}$).

Monitoring for all possible contaminates is both financially not feasible and physically impossible within the current resource levels under which water companies operate. An approach applied by water companies to overcome this potential problem is to undertake a risk management strategy, where the likelihood and the consequence of contamination are calculated for each possible contaminant to determine its risk of contamination (Hamilton *et al.*, 2003; O'Connor, 2005 &, 2008). From the risk assessment, all known possible contaminants identified are prioritized and a monitoring program is developed, with the frequency of monitoring being dictated and limited by the costs of monitoring and analysis of samples, which can be upward of AUD500 for each sample per contaminant (Personal Communication; Considine, 2005).

In 2009, the Southern Nevada Water Authority published results of an extensive study on the quality of water (from source water, finished drinking water, and water within the distribution system (*i.e.,* tap water)) from 19 water companies providing water to more than 28 million people throughout the USA during 2006 and 2007 (Benotti *et al.*, 2009). The most frequently detected compounds found were atenolol (cardiovascular pharmaceutical), atrazine (pesticide), carbamazepine (pharmaceutical), estrone (hormone), gemfibrozil (pharmaceutical), meprobamate (carbamate derivative), naproxen (pharmaceutical), phenytoin (pharmaceutical), sulfamethoxazole (antibiotic), TCEP (pharmaceutical), and trimethoprim (antibiotic) (Benotti *et al.*, 2009). Atrazine, meprobamate and phenytoin were detected in more than half of the finished water and tap water samples, at concentrations up to 0.93 μg $L^{-1}$. Also surprisingly, atrazine was detected in the source water feeding into almost all drinking water treatment plants investigated, including those areas where atrazine was not used agriculturally.

Similarly, Salina (2008) portrayed the current predicament our communities face in terms of water quality and water availability. She reported that more than 116,000 anthropogenic chemicals find their way into public water supply systems globally, with estimates ranging from five hundred thousand to seven million people becoming ill per year from drinking contaminated tap water. Atrazine and other pesticides were identified as the possible cause for increased cancer rates, birth defects and decreased sperm production amongst males in Tasmania and regions of the USA (Salina, 2008).

Given the level of scrutiny the water sector operates under (namely water quality, availability and pricing), robust techniques are needed for the determination of pesticide residues in source waters and water within the distribution system that are both cost effective and reliable. The aim of this project was to develop a sensor that provides immediate feedback on the concentration of a number of potential pesticide residues and their degradation products based on the principles of FIA.

A feature of all FIA is the ability to select an appropriate chemical reaction and manipulate parameters to yield a response from a specific analyte within the sample matrix without batch sample treatment or derivatisation. In many cases pre concentration is also unnecessary.

In order to fulfill the aim of this research, a series of tasks and experiments were proposed and carried out, namely to:

1. construct a flow injection system suitable for the determination of pesticides in water
2. optimise the flow injection parameters
3. validate the system against a recognized alternative method
4. apply the developed instrument to the determination of pesticide residues in natural waters
5. evaluate the chemiluminescence detection system for its suitability as a portable instrument for the determination of pesticide residue in drinking water

## 1.2. Document Structure

This thesis is presented in ten chapters, in addition to this introductory chapter:

- **Chapter 2** gives an overview to the pesticides used in this study, how they are used by society, and where they are found within the environment. A description of the effects pesticide contamination have on flora and fauna is given, as well as the common issues associated with pesticide monitoring, and the current analytical techniques and methods used for pesticide detection and identification. In presenting the methods currently utilised for pesticide determination, a new alternative method based on FIA is presented.

- **Chapter 3** details all the chemicals, reagents, methods and equipment used to complete all the experimental work detailed in the subsequent chapters.
- **Chapter 4** details the construction and optimisation of a flow injection method for the rapid determination of atrazine residue, with a statistical comparison of the analytical figures of merit for the proposed FIA method and a recognised direct injection high performance liquid chromatography (HPLC) method.
- **Chapter 5** details the work carried out to further refine and optimise the HPLC validation method.
- **Chapter 6** compares the suitability of two flow injection reagents for the determination of organophosphates (dicrotophos and monocrotophos) in natural waters; limits of detection are established along with a statistical comparison with a standard analytical method and values cited from the literature.
- **Chapter 7** investigates the application of the FIA method to natural waters, with an investigation into the effect of dissolved organic matter (measured as dissolved organic carbon (DOM)) utilising attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR).
- **Chapter 8** demonstrates a semi/fully automated system that incorporates an in-line solid phase extraction (SPE) procedure for the removal of interfering species for the rapid analysis of pesticide residues in natural waters, with an analytical comparison with a modified direct injection HPLC method described in Chapter 5.
- **Chapter 9** details the in-line separation and differentiation between multiple pesticides and selected metabolites for the rapid determination of pesticide residues in natural water samples, with an analytical comparison with a HPLC method.
- **Chapter 10** further investigates the interaction between DOM in natural waters and the selected pesticide residues using three dimensional excitation emission matrix (3D EEM) Fluorescence.
- **Chapter 11** summarises the conclusions of this study, and proposes recommendations and areas of further research into the use of the current instrument for the continuous monitoring of pesticide residues in matrices such as wastewaters, with potential application to other pesticides of concern and within treatment operations.

# CHAPTER TWO: LITERATURE REVIEW

*"Pesticides, those 'super chemicals' used to control pests around the home as well as in agriculture, have a prominent place in the day-to-day activities of our technologically advanced society. They can't be ignored, and they won't go away. Like it or not, we are living in the 'The Chemical Age"*.

— **G Ware, "*The Pesticide book*", 1978 (1st Edition).**

Human beings, as if by nature, have strived to mitigate the effects of insects and other pests over the centuries. Throughout history, people have utilised various types of pesticides to combat pests such as insects, weeds, bacteria, rodents, and other organisms. Srinivasan (2003) described Homer (1,000 BC) writing about the use of sulfur in an effort to combat pests in food crops; the ancient Romans were renowned for applying salt to the crops of their enemies to bring on famine. Ware (2000) described olive oil applied on crops to control diseases, and the Chinese used mercury and arsenic compounds to control body lice and other pests. Bordeaux mixture (a combination of copper sulfate, lime and water) was first used in the nineteenth century, and is still heavily used in viticulture for protection from mildew (Ware, 2000). It was at this time that the scientific use and understanding of pesticides really began to develop. However, it wasn't until the early twentieth century that human beings significantly increased their dependence on pesticides (Dixon, 2004).

Although pesticide use is not new, the chemical substances used as pesticides have changed. The first documented evidence of pesticide use involved readily available inorganic chemicals such as: sulfur as a fungicide, copper as an algaecide, lead and arsenic as insecticides, and chromium, copper, and arsenic as wood preservatives (Srinivasan, 2003). It wasn't until the twentieth century that inorganic pesticides were replaced with synthetic organic compounds for use as pesticides. It is noteworthy that the use of inorganic chemicals as pesticides is still common practice today.

In the 1930s, the commercial production of dinitrophenol (DNOC) and dithiocarbamate began and the insecticide dichloro diphenyl trichloroethane (DDT) came into use during World War II (1939) (Ware, 2000). DDT was seen by the global community to be a new

labour saving technique to maintain public health and improve agriculture (applied as an insecticide to agricultural crops from the 1950s onwards), and its use continued to grow despite some well-documented environmental and social disasters (Mellanby, 1992). It would take another thirty years for the creation of the US Environmental Protection Agency (USEPA) (1970) and for the use of pesticides to start to be monitored (USEPA, 2008b). Since the inception of the USEPA, there have been numerous advances in pesticide development. Many pesticides that were used in the 40s, 50s and 60s have since been either banned or regulated as a result of obtaining a greater understanding of previously unknown subtle, and often potentially long term effects, that these chemicals have on the environment, non target organisms and/or human health. The USEPA has continued to closely monitor the use of pesticides. This has led to a boom in new pesticide formulations with different application methods and modes of action that comply with the strict environmental regulations imposed by the USEPA and other government organisations and departments (USEPA, 2008b). The continuous development of pesticide formulations has created more selective, less persistent and less toxic pesticides. Currently there are over 900 registered pesticides used throughout the world; between 1999 and 2002, the USEPA alone added 75 new pesticides to the list of registered pesticides of which more than half were later upgraded from "conventional chemicals" to "potential risk" pesticides (USEPA, 2008b).

## 2.1. Pesticides in Society

A pesticide by definition, '*is an agent, substance or mixture of substances employed to destroy, mitigate, repel or control pests and includes insecticides, herbicides, fungicides and other controlling materials'* (Oxford Dictionary, 1996). Pests are defined as organisms that are detrimental to human beings or their interests in some manner (Oxford Dictionary, 1996); they are defined by humans and their lifestyle or preference, not by nature. Every human being has encountered a pest or its consequences (whether it be a farmer, home owner, gardener, or outdoor adventurer) and thereby intuitively has an understanding of them, and the chemicals used to mitigate their existence (Ware, 2000).

Pesticides have become an integral part of today's society; they are relied upon to combat unwanted organisms. Ware (2000) collated data on pesticide use and at the time of publication it was estimated that there are in excess of 100,000 diseases/pests (viruses, micro-organisms or other plants) affecting plants that are depended upon for the survival of humans and animals. More specifically, it is estimated that there are 30,000 weed species

that compete with food crops; 18,000 of which cause serious economic loss. Similarly, it has been estimated that there are more than 1,000,000 species of insects, of which 10,000 contribute to crop destruction. The recent push for US farmers to increase their maize and soy crops for the production of bio-fuels has resulted in a significant change in the surrounding landscape; maize and soy crops have expanded by 19%, dramatically decreasing plant diversity. Researchers have found that due to the change in diversity, the populations of problematic insects (*e.g.* soybean aphids) have increased significantly, whereas the populations of insects that usually prey on them have remained stable, resulting in more pesticide loads being applied to crops in an attempt to mitigate the growing pest population (MacKenzie, 2008).

Each year millions of people die or are seriously disabled by diseases, insects, vermin and weeds at a cost of 100 billion dollars annually ($US) (Ware, 2000). The need to protect crops from pests simply isn't just an economic driver; it is also a humanitarian obligation. At the turn of the twentieth century the global population reached 6 billion people; it is estimated that by 2025 the population will be in excess of 8 billion people (Department of the Environment and Heritage (DE&H), 2006). Pesticides have become indispensable and are needed to maintain high crop yields so that food supplies, textile production and human health standards can be met for a growing population.

There are two key factors that have influenced how pesticides are used today: advances in science and expansion of a free global market. Today we know more of the effects and consequences of pesticides in the global community than ever before and are continuing to expand that knowledge base (Radcliff, 2002; Rushworth, 2004; DE&H, 2006). Global communities are now free to trade on a scale never before seen with few or often no restrictions, which drives the need for higher crop yields in order to meet an increase in demand (Abaza, 1999).

It is the global competition for fresh produce that pushes farmers to strive to obtain maximum yields from crops with less labour-intensive solutions to maximize profits. Previously, developments in agriculture came via the industrial revolution with steam and combustion engines leading to advancements in mechanical harvesters, new crop cultivators and refined petroleum products (Ware, 2000). The industrial revolution brought a hunger for new technology to aid industries to produce more food for a growing population.

However, with the increased production, new land tillage and large scale monoculture, the need to mitigate pests has become more urgent and widespread (DE&H, 2006).

The manufacture of pesticides is a multi billion-dollar industry (55 billion dollars as of 2009). The United States alone has an annual market of USD 14.1 billion per annum, while Australia spends approximately a tenth of that amount (Worm & Vaupel, 2009). Although domestic pesticide use has declined, pesticide production is increasing due to higher demands from agriculture, industry and government. It is estimated that 79% of the pesticide market is for agriculture, 13% for industry and government, and 8% for domestic use (Ware, 2000). If pesticides were not used, it is estimated that one third of the world's food crops would be destroyed by pests either during growth, harvesting, or storage. Losses would be even higher in developing countries (Dixon, 2004).

However, with pesticide production increasing, and pesticides being applied on a larger scale, it is no surprise that pesticide contamination has been widely publicised over the past 30 years and will continue to be a point of contention into the future (Radcliff, 2002; Davis, 2004; Palma *et al.*, 2004; Rushworth, 2004; Tariq *et al.*, 2004; DE&H, 2006).

## 2.2. Pesticides in the Natural Environment

There are numerous accounts of pesticide contamination within the natural environment; we have a good understanding of where they are being used and their application rates. Understanding how pesticides move from the point of application has been the focus for many researchers. For example, Zhu & Li (2002) described the effect of 30 years application of bromacil and hexazinone on pineapple crops in Hawaii and illustrated the higher mobility of bromacil down and across the soil profile. Oliver *et al.* (2003a, 2003b) investigated the effects of land use and the sorption characteristics of fenamiphos and atrazine (including atrazine metabolites), and Dousset *et al.* (2004) monitored the movement of hexazinone and glyphosate through soil columns under plantations. Graymore *et al.* (2001) presented a review on the impacts atrazine has on waterways and concluded that current regulations on application rates of atrazine were insufficient protection for aquatic ecosystems. The chemical parameters and characteristics used to describe the mobility and persistence of pesticides in the natural environment continues to be widely researched and debated (Mansur & Feicht, 1994; Wittmann & Schmid, 1994; Kookana *et al.*, 1995; Mansour *et al.*, 1997; Ueoka *et al.*, 1997; Franzmann *et al.*, 1998; Kookana *et al.*, 1998; Zambonin & Palmisano, 2000; Zhu & Li, 2002; Oliver *et al.*, 2003a; Oliver *et al.*, 2003b; Davis, 2004;

Dousset *et al.*, 2004; Palma *et al.*, 2004; Rushworth, 2004; Tariq *et al.*, 2004; DE&H, 2006; Lewis *et al.*, 2009).

Understanding how pesticides work to restrict the impact of pest species, how mobile they are through soils and how persistent they are in the environment, is important; having a better understanding of such characteristics aids users in better manage pesticide applications (*e.g.* when and how) to limit their migration from the point of application.

### 2.2.1. Pesticide Mode of Action

Pesticides are chemicals that inhibit or interrupt normal growth and development of target species. They are widely used today for various applications as previously described. The two classes of pesticide that have been selected for this study are two triazines (and by extension one triazinone, all of which are a class of herbicide) and two organophosphates (a class of insecticide); for obvious reasons each pesticide type has a unique mode of action resulting from the difference in target species.

Triazine herbicides are classified according to their selectivity (non selective, grass control, broadleaf, etc.), time of application (post emergence or pre emergence), translocation in the plant, persistence and/or site of action (Ware, 2000).

Organophosphate insecticides are classified according to their associated functional groups (or derivatives): aliphatic, phenyl, or heterocyclic, and by their selective mode of action: attack the nervous system, inhibit growth and development, or inhibit energy production (Ware, 2000).

#### *Triazine pesticides*

The herbicides selected for this study are the triazines atrazine and simazine, and the triazinone hexazinone. Triazine and triazinone herbicides are post emergence pesticides used for broadleaf and grass control. They inhibit photosynthesis by targeting photosystem II (Ware, 2000).

Triazines and triazinones are absorbed through the roots and shoots of plants, and are translocated outside the plant cell plasma membrane (apoplastically) through the xylem (where the xylem is plant vascular tissue that transport nutrients, energy and water (Raven & Johnson, 1992)).

Peterson *et al.* (2001) described the action of triazines and triazinones as inhibiting normal photosysnthesis by blocking electron transport at the quinone electron carrier

site at Photosystem II. This blockage results in chlorophyll destruction, limits photosynthesis and leads to a build-up of $CO_2$.

### *Organophosphate pesticides*

The organophosphates selected for this study are monocrotophos and dicrotophos. They are aliphatic organophosphates used to control flea hoppers, aphids, thrip, stink bugs and plant sucking insects (USEPA, 1999).

Aliphatic organophosphates are systemic insecticides (Ware, 2000); they are absorbed through roots and shoots in plants, and are translocated through the xylem to the above ground parts of plants where they are toxic to any sucking insects feeding on the plant juices.

Brown (2005) described the mode of action of organophosphate pesticides, including carbamates, as inhibiting cholinesterase enzymes that are an important part of the nervous system. This inhibition results in the accumulation of acetylcholine (a neurotransmitter) which interferes with the neuromuscular junction, producing rapid twitching of voluntary muscles and finally paralysis.

## 2.2.2. Pesticide Mobility and Persistence

Once a pesticide has been introduced into the environment, its chemical and physical properties determine its fate: where it goes and how long it lasts (Radcliff, 2002). Each pesticide has its unique set of properties. Pesticides are designed to last long enough to do their primary job of controlling undesirable pests, then to break down to non-toxic substances. However, pesticide persistence is highly variable since the rate at which pesticides break down not only depends on the pesticide's chemistry, but also surrounding environmental factors, such as sunlight, temperature, rainfall and soil pH (Radcliff, 2002). Pesticides that break down quickly do not offer much opportunity for exposure.

Pesticide mobility is affected by the pesticide's sorption, water solubility, and vapor pressure. Mobility is also influenced by environmental and site characteristics including weather, topography, canopy, ground cover, soil organic matter, texture, and structure. The chemical and physical characteristics of a pesticide, along with the properties of the surrounding environment and application method, all combine to influence the redistribution/mobility of a pesticide at the application site or migration off site. After application, a pesticide may either:

- be attached to soil particles, vegetation, or other surfaces and remain near the site of application
- be attached to soil particles and move with eroded soil in runoff or wind
- dissolve in water and be taken up by plants, or leach into aquifers, waterways or the ocean (regarded as a major problem in areas of high rainfall or regions susceptible to frequent flash flooding (Arnold *et al.*, 1990)
- volatilize or erode from foliage or soil with wind and become airborne

The pesticide half-life, the soil sorption coefficient, water solubility, and vapor pressure all aid in providing information on the potential environmental fate of pesticides. These parameters are used to calculate the Groundwater Ubiquity Score (GUS), which can be used to predict the likely fate of a specific pesticide (Gustafson, 1988; Fishel, 2006).

Table 2-1 and Table 2-2 illustrate the chemical and physical parameters used to determine the persistence and mobility of pesticides, and the properties of the selected pesticides used for this study respectively.

**Table 2-1: Chemical and physical properties used to determine the persistence and mobility of pesticides**

| Parameter | Unit | Definition | Interpretation |
|---|---|---|---|
| Distribution Coefficient | $K_d$ | Indicates a pesticide's ability to either stay in solution or bind to soil particles (soil specific). | A low $K_d$ indicates the pesticide is more likely to remain soluble, A high $K_d$ indicates the pesticide is more strongly bound to soil particles. |
| Sorption Coefficient | $K_{OC}$ | $K_{oc}$ is the distribution coefficient divided by the amount of organic carbon in the soil. | The higher the $K_{oc}$ value, the more strongly the pesticide is bound, and therefore, the less mobile it is. |
| Half Life | $t_{1/2}$ | The amount of time it takes for the concentration to halve | Pesticides can be divided into three categories based on half-lives: non-persistent pesticides with a typical soil half-life of less than 30 days, moderately persistent pesticides with a typical soil half-life of 30 to 100 days, or persistent pesticides with a typical soil half-life of more than 100 days. |
| Groundwater Ubiquity Score | GUS | The GUS is a number that relates pesticide persistence (half-life) and sorption ($K_{oc}$) in soil.<br><br>$GUS = \log_{10}(t_{1/2}) \times (4 - \log_{10}(K_{OC}))$.<br>(Gustafson, 1988) | Low GUS score, <2 moderate to low risk of groundwater contamination. High GS score, >3 high to very high risk of groundwater contamination. |
| Water Solubility | mg $L^{-1}$ or ppm | Amount of pesticide that is soluble in water. | Solubility values are used to compare compounds and are significantly affected by additives (*e.g.* surfactants) that are added to increase/decrease solubility. NOTE:- Solubility of those pesticides that are weak acids or bases is also influenced by pH. |
| Volatilisation | $K_h$ | Henrys Law constant. $K_h$ is defined as the concentration of pesticide in air divided by the concentration in water. | The higher the Henry's Law constant, the more likely a pesticide will volatilize from moist soil. A Henry's Law index values of <100 has a low potential to volatilise from moist soil. Pesticides with Henry's Law index values > 10,000 have a high potential to volatilise. |
| Vapour Pressure | mm Hg at 20 degrees (C) | Ability to transfer from soil, water or plant surface into the atmosphere as either a vapour or gas. | The higher the vapour pressure, the more likely a pesticide will volatilize into the atmosphere. Vapour pressure values of >1,000 have a high potential to volatilise. Pesticides with vapour pressure values <10 have a low potential to volatilise. |

**Table 2-2: Chemical properties of selected pesticides**

| Pesticide Name | Class | Type | Molecular Weight (MW) (g $mole^{-1}$) | $K_{OC}$ (g $ml^{-1}$) | Half-Life (days) | Water Solubility (mg $L^{-1}$) | Vapour Pressure (mm Hg at 20 degrees (C)) | Kh (atm-$m^3$ $mol^{-1}$) | GUS score | GUS Rating |
|---|---|---|---|---|---|---|---|---|---|---|
| Atrazine (EXTONET, 1996a) | Triazine | Herbicide | 215.7 | 100 | 53 (pH=4.8)<br>113 (pH=6.5) | 28 | $3.0 \times 10^{-7}$ | $2.63 \times 10^{-7}$ | 3.5 | High |
| Atrazine-desethyl | Triazine | Metabolite | 187.6 | NA | 150 (soil) | 438 | NA | NA | NA | NA |
| Atrazine-2-hydroxy | Triazine | Metabolite | 197.2 | NA | NA | 47 | NA | NA | NA | NA |
| Atrazine-desisopropyl | Triazine | Metabolite | 173.6 | NA | 150 (soil) | 114 | NA | NA | NA | NA |
| N-Isopropylammelide | Triazine | Metabolite | 170.1 | NA | NA | NA | $3 \times 10^{-7}$ | NA | NA | NA |
| Cyanuric acid | Triazine | Metabolite | 129.1 | NA | NA | 26,000 | NA | NA | NA | NA |
| Biuret | Triazine | Metabolite | 103.1 | NA | NA | NA | NA | NA | NA | NA |
| Simazine (EXTONET, 1996c) | Triazine | Herbicide | 201.7 | 138 | 100 (pH=7) | 5 | $6.1 \times 10^{-9}$ | $4.6 \times 10^{-10}$ | 2.55 | Moderate |
| Hexazinone (EXTONET, 1996b) | Triazinone | Herbicide | 252.3 | 610 | 139 (pH=7) | 33,000 | $2.0 \times 10^{-7}$ | $2.1 \times 10^{-12}$ | 4.6 | Very high |
| Monocrotophos (EXTONET, 1995b) | Organophosphate | Insecticide | 223.2 | 0.9 - 30.8 | 30 (pH=7) | Soluble | $2.2 \times 10^{-6}$ | $6.5 \times 10^{-13}$ | 5.8 | Very high |
| Dicrotophos (EXTONET, 1995a) | Organophosphate | Insecticide | 237.2 | 104 - 227 | 117 (pH=5)<br>72 (pH=7) | Miscible | $1 \times 10^{-4}$ | $5.1 \times 10^{-6}$ | 3.1 | High |

***Note:*** *NA – not available in the literature.*

All the selected pesticides are considered either soluble in water or have fairly high solubility in water, this is not taking into account the additives in pesticide formulations such as surfactants which significantly increase the formulation solubility. The half-lives for the triazines are all over 100 days at neutral pH: those for monocrotophos and dicrotophos are 30 and 70 days, respectively. This indicates that the triazines have a much longer life expectancy after application and have a higher potential to be persistent in the natural environment than either monocrotophos or dicrotophos. The sorption coefficient ($K_{OC}$) for monocrotophos is 0.8 – 30.8 (dependent upon $K_d$), indicating that it is not likely to be strongly attached to soil particles. Atrazine and simazine have $K_{OC}$ values of 100 and 138 respectively, indicating that they are likely to be more strongly bound to soil particles. Dicrotophos ($K_{OC}$ of 104 -227) and hexazinone ($K_{OC}$ of 610) has an even stronger attraction to soil particles and a tendency to remain within the soil profile, although this is not always the case as evidenced the water solubility coefficient and GUS rating (and its documented contamination in groundwater) (Radcliff, 2002). The selected pesticides all have a low vapor pressure and volatilisation coefficient (Henrys Law constant, $K_h$), indicating their potential to volatilise is low. All the pesticides have GUS scores above 3 with the exception of simazine. This would suggest the probability of the selected pesticides becoming 'mobile' and reaching a waterway is relatively high; this is supported by their frequent detection within the environment (Davies *et al.*, 1994; Korth, 1995; Kookana *et al.*, 1998; Barnes & Holz, 1999; Haynes *et al.*, 2000; Amis, 2008; Lewis *et al.*, 2009).

### 2.2.3. Pesticide Metabolites

It is understood that pesticides degrade through microbial activity, chemical activity, or the action of sunlight (Radcliff, 2002). All three processes may participate in the breakdown of a single pesticide, the rate of degradation being dependent on its chemistry and prevailing environmental conditions (Radcliff, 2002). Ultimately, the degradation products of any organic chemical will be water, carbon dioxide, and minerals; however, the intermediate metabolites of some pesticides, in particular atrazine, are of concern for both human health and the environment. Metabolites are generally less biologically active than the parent compound and occur at lower concentrations; however, atrazine metabolites have been documented to cause premature aging, affect growth and development, affect reproductive function, and delay puberty in a variety of species (Cooper, 2008).

Theoretically, pesticide metabolites degrade more rapidly than the parent compound, appearing in the environment as only intermediate compounds en route to the final degradation products. Previously, the focus has not been on pesticide metabolites but primarily on the parent compounds. The advancement in analytical techniques for pesticides in the environment has enabled researchers and analysts to expand their list of target analytes to include pesticide metabolites. Interestingly, researchers have found that in some cases pesticide metabolites remain in the environment far longer than its original parent compound. For example, Guzzella *et al.* (2006) detected levels of atrazine metabolites (atrazine-desisopropyl, atrazine-desethyl and atrazine-2-hydroxy) equal to or greater than the detected level of atrazine in groundwater samples.

The atrazine metabolites atrazine-desethyl, atrazine-2-hydroxy, atrazine-desisopropyl, N-isopropylaminelide, cyanuric acid and biuret were included in this study; their chemical properties are listed in Table 2-2, although these data are limited.

### 2.2.4. Pesticides in the Australian Environment

There have been several reviews on pesticide contamination in surface, ground and irrigation waters throughout Australia (Kookana *et al.*, 1998; Radcliff, 2002; Amis, 2008). Figure 2-1 summarises their findings for atrazine, simazine, hexazinone and atrazine metabolite contamination along with some more recent literature (a detailed summary of each record is located in Appendix A for completeness). There has been no recorded contamination with either monocrotophos or dicrotophos in Australia. However, it is noted that monocrotophos was recently reviewed by the Australian Pesticides and Veterinary Medicines Authority (APVMA, 2011); it was concluded that although the level of efficacy required to meet current registration standards was maintained, the use of monocrotophos was reduced.

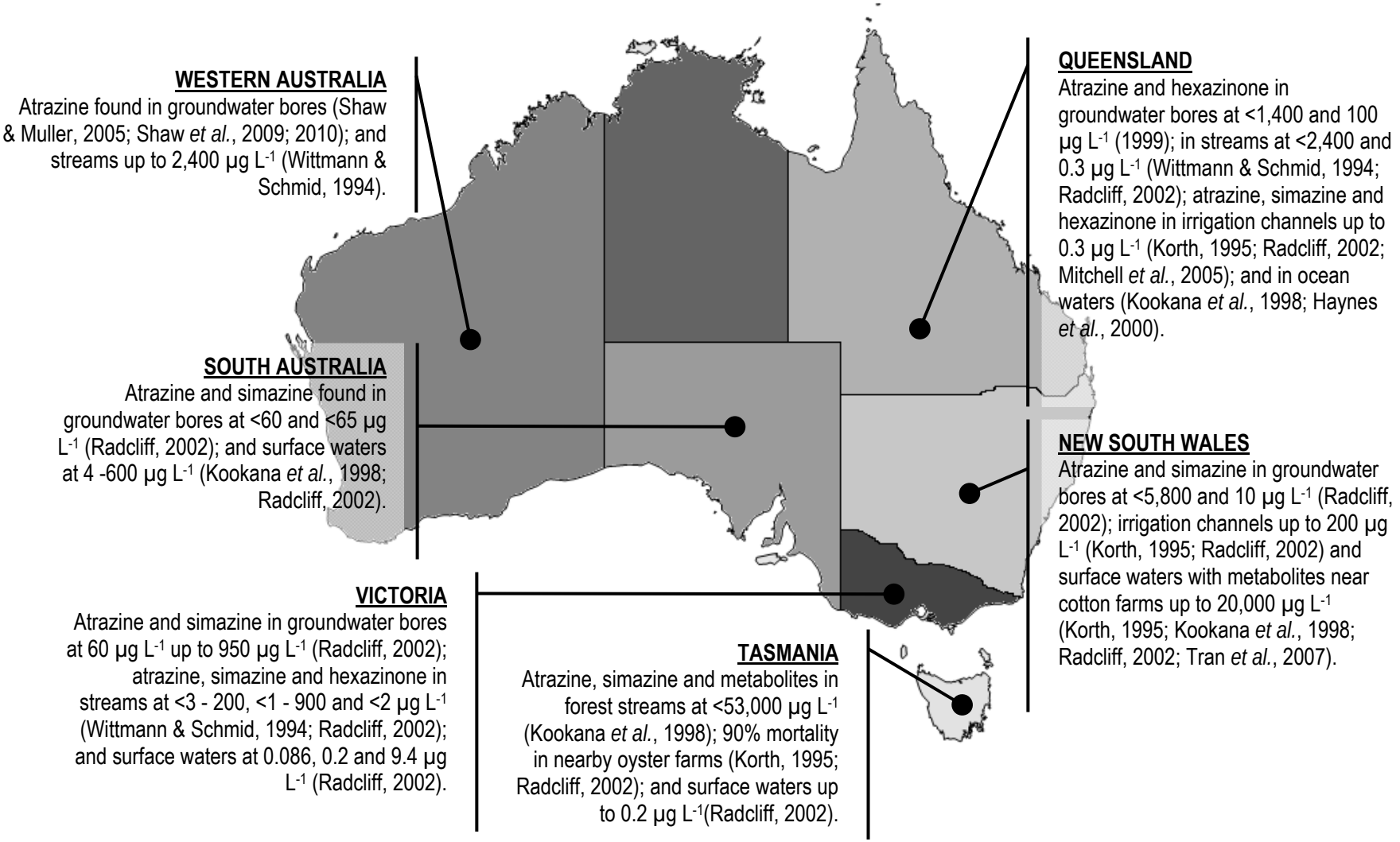



**Figure 2-1: Pesticide contamination in Australian waterways.**

### *Surface waters within agricultural areas*

Anecdotal data from research papers (Australia) surveyed indicate a rising trend in the number of pesticides detected/recorded over the years 1991-2009, with the incidence of atrazine and hexazinone appearing to be steadily increasing (Radcliff, 2002). Storm events can substantially increase both the concentration and load of pesticides in storm surface waters, as highlighted in an incident in Namoi (New South Wales (NSW), Australia). Significant loads of atrazine were transported off cotton farms within the Namoi catchment into the surrounding river system during an extended storm in July 1993. The concentration of atrazine exported by runoff during the storm peaked at 2.25 mg $L^{-1}$ in surface waters. It is worth noting that atrazine had not been reported to have been used 12 months prior to the storm event (Radcliff, 2002). More recently Lewis *et al.* estimated 1.5 tonnes of atrazine was washed off agricultural lands into rivers leading out to the Great Barrier Reef during a storm event (Denholm, 2008; Lewis *et al.*, 2009). In addition, the effects of the recent Queensland floods (2011) are already evident. Shortly after the floods in south east Queensland, visible plumes of polluted water were observed gushing from many Queensland rivers into the Great Barrier Reef. The full extent of the event and the impact of contaminants (comprising pesticide residues) on the reef will take several years to be fully realised (Crow, 2011; Pyett, 2011; Thomas, 2011)

### *Irrigation areas*

It is not uncommon for large quantities of herbicides (>100,000 kg) to be applied to irrigation areas, in particular across south-western NSW for the growing of rice. Bensulfuron-methyl is the primary pesticide used on most rice crops; however, other herbicides are used for general weed control and seedbed preparation such as glyphosate, diquat, paraquat, atrazine and diuron. Generally, supply water from the rivers in this region are typically of high quality and few pesticides were detected (Kookana *et al.*, 1998). However, reported pesticide levels in irrigation channels throughout Australia vary in concentration (<200 µ $L^{-1}$) (Korth, 1995; Kookana *et al.*, 1998; Haynes *et al.*, 2000; Muller *et al.*, 2000; Tran *et al.*, 2007). Atrazine was detected in most drains during the season of application, and levels often exceeded guidelines both for drinking water and protection of the aquatic environment (Radcliff, 2002).

### *Waterways*

In Tasmania, between 1989 and 1992, triazine herbicides atrazine and simazine were detected in 20 out of 29 streams sampled that drained forestry and agricultural catchments (Davies *et al.*, 1994; Radcliff, 2002). Forest spraying had been carried out by helicopter with relatively high application rates. Concentrations of herbicides ranged over several orders of magnitude; the highest concentration of atrazine was 53 mg $L^{-1}$ (53,000 μg $L^{-1}$) and of simazine 478 μg $L^{-1}$. Atrazine residues decreased with time after spraying from 8.1 mg $L^{-1}$ on the day of spraying to 0.3 mg $L^{-1}$ around 13-15 months later (Davies *et al.*, 1994; Radcliff, 2002). The theoretical half-life of atrazine in these Tasmanian streams was calculated to be approximately 3 months (90 days) which is comparable to the typical half-life of approximately 113 days (pH 6.5) reported in the literature (Radcliff, 2002).

More recently in 2004, an aerial pesticide sprayer crashed in northern Tasmania releasing its pesticide load of atrazine, simazine, and alphacypermethrin (a pyrethroid insecticide) into the surrounding built and natural environment. This resulted in 90% mortality at a nearby oyster farm along with an observed increase in health complaints from the local community (Rushworth, 2004); the effects of pesticide exposure is an ongoing concern for Tasmanian communities (Knox, 2010). However, a 2010 report concluded that pesticides are not to blame for the observed increase in health complaints in Tasmania, which are most likely due to a combination of environmental stresses (Bately *et al.*, 2010).

### *Groundwater*

As illustrated in Table 2-2, triazine herbicides have a low ability to bind to soils and therefore are considered relatively mobile. It is then no surprise that triazines are often found in groundwater systems in rural regions of Australia. The National Health & Medical Research Council (NHMRC–NRMMC, 2004) described atrazine as one of the most widely used herbicides in Australian agriculture, with high potential to contaminate ground and surface water, and narrow safety margins for aquatic organisms. The NHMRC–NRMMC (2004) proposed measures to monitor and reduce atrazine contamination of aquatic systems, particularly to eliminate poor agricultural practices. Simazine may also occur in groundwater but is not as mobile as atrazine (DE&H, 2006).

In 1992 an extensive groundwater survey found that triazine herbicides (most commonly atrazine) were the most frequently detected. In some areas as many as 80% of bores contained detectable residues of atrazine and/or simazine (including their metabolites)

(Kookana *et al.*, 1998; Radcliff, 2002). Many of the groundwater systems contaminated with triazines (and to a larger extent pesticides in general) could be linked to agricultural activities that used the detected pesticides within the aquifer recharge zone.

### 2.2.5. Australian Drinking Water Guidelines (ADWG) for pesticide residue

The Australian Drinking Water Guidelines (NHMRC–NRMMC, 2004) provide 'health trigger values' and recommended 'guideline values' for 55 pesticides from a range of classes. These are designed to protect freshwater ecosystems from adverse environmental and health related effects from chronic exposure, but the majority of guideline values are set at minimum levels of detection.

The trigger values for the selected pesticides used in this study are detailed in Table 2-3.

**Table 2-3: Selected Australian Drinking Water Guideline pesticide trigger values.**

| Pesticide* | Guideline Value[a] (mg $L^{-1}$) | Health Trigger Value[b] (mg $L^{-1}$) |
|---|---|---|
| Atrazine[c] | 0.0001 | 0.04 |
| Simazine | 0.0005 | 0.02 |
| Hexazinone[c] | 0.002 | 0.3 |
| Monocrotophos | - | 0.001 |
| Dicrotophos | - | - |

***NOTE:*** *[a]These are generally based on the analytical limit of detection. If a pesticide is detected at or above this value the source should be identified and action should be taken to prevent further contamination; [b]Based on 10% of acceptable daily intake; [c]These pesticides have either been detected on occasions in Australian drinking water or their likely use would indicate that they may occasionally be detected. *Routine monitoring for pesticides is not required unless potential exists for contamination of water supplies (NHMRC–NRMMC, 2004).*

The mobility and persistence of atrazine and hexazinone have led to their being labeled as 'pesticides of concern' in drinking water; the current Australian Drinking Water Guidelines (2004) state that atrazine and hexazinone should not exceed 0.1 µg $L^{-1}$ and 2 µg $L^{-1}$ respectively; and in the event of a major contamination of a catchment area, health trigger values of 40 µg $L^{-1}$ and 300 µg $L^{-1}$, respectively, have been set. The latest draft of the new water guidelines lowered the allowable amount of atrazine in drinking water from the 1998 guideline value of 0.5 µg $L^{-1}$ to the current limit of 0.1 µg $L^{-1}$, while the health trigger value increased from 20 µg $L^{-1}$ to 40 µg $L^{-1}$ (NHMRC–NRMMC, 2004). The drinking water guideline value has decreased due to improvements in detection limits, while the health trigger value has increased due to the statistical probability of atrazine contamination in a drinking water catchment being reduced. The health trigger values are set to assist the health authorities in managing a spill or severe case of misuse. The guidelines assume that if a pesticide is

detected, steps would be taken to eliminate the source and remove it (*e.g.* via granular activated carbon) (NHMRC–NRMMC, 2004). New research in the US indicates that exposure to atrazine below the Australian drinking water health guidelines (*i.e.,* 20 μg $L^{-1}$) is detrimental to human health, causing feminisation of juveniles and the disruption of human placental cells (Denholm, 2008; Morris, 2008b). Previously, atrazine and simazine have been determined to have short-term exposure symptoms of congestion of the heart, lungs and kidneys, low blood pressure, muscular spasms and weight loss; the long-term exposure symptoms are cardiovascular damage, retinal and muscular degeneration, and cancer (NHMRC–NRMMC, 2004). Hexazinone is considered a class D carcinogen by the US EPA (may cause cancer in humans – not verified)(USEPA, 2008b), while monocrotophos and dicrotophos are both considered extremely toxic substances affecting the nervous system; symptoms consist of excessive sweating, headache, weakness, giddiness, nausea, vomiting, hypersalivation, abdominal cramps, diarrhoea, blurred vision and slurred speech (EXTONET, 1995b &, 1995a &, 1996a &, 1996c &, 1996b; EPA, 1999; 2007;Pullin *et al.*, 2007; APVMA, 2011).

Considerable effort has gone into limiting the use of atrazine within drinking water catchment recharge zones and similar work is currently underway for hexazinone and simazine (Personal Communication, Considine 2005). To date, no Australian drinking water supply (*i.e.* customer tap water) has been reported to be affected by atrazine, simazine, hexazinone, or monocrotophos above guideline values. Where these pesticides have been detected in source waters, the water supply has been removed from the distribution network. In 2008, Hobart Water (Tasmania) reported atrazine below guideline values in the Clyde and Derwent Rivers, source water to Hobart's Bryn Estyn Water Treatment Plant (Morris, 2008a). It is reported that the investigation into the incident was concluded without identifying the actual source of contamination, without comprehensively testing the water supply which is sourced directly from the Clyde, and without notifying any of the communities drawing their water from the Clyde and the Derwent rivers directly (Morris, 2008a). Barwon Water (Victoria, 2005) experienced a similar incident with hexazinone detection in a waterway that is used to source drinking water; however, action was taken quickly to shut off the source, even though the levels detected were below guideline values (both for health and recommended guidelines), and to identify the source of contamination, which was found to be a nearby pine plantation. To date, Barwon water is still working closely with the management of the plantation to minimise a recurrence. Interestingly, to

the knowledge of Barwon Water, hexazinone has not been used in the area since the contamination was first highlighted to the water company but continues to be occasionally detected in the source water (Personal Communication, Buchanan 2008).

In 2008, the AP&VMA announced its review of atrazine had concluded that '*no changes to the existing health standards were needed*' (Denholm, 2008). In contrast, atrazine has been banned in many European Union states, including Switzerland where atrazine is manufactured, due to the risks to the environment and human health (Salina, 2008).

Simazine and hexazinone are flagged as priority two pesticides, which are pesticides that will be subject to reviews in the future based on environmental and human health effects relating to their use (Australian Pesticides and Veterinary Medicines Authority (AP&VMA, 2001). (As of 1/3/2011 the reviews of hexazinone and simazine have not been completed)

### *Adequacy of environmental monitoring in Australia*

The monitoring of pesticide residues in the Australian environment varies greatly, depending on the State or region, the industry and the environmental medium (DE&H, 2001 &, 2006). The limited amount of monitoring of pesticide residues and its variability from one region to another prevents a clear understanding of pesticide exposure in the Australian environment as illustrated in Figure 2-2 (Table A-1), although there are extensive data. There can be a tendency for the extent of monitoring programs to be determined by the cost of monitoring rather than the basis of risk appraised (Personal Communication; Considine, 2005). In 2008, almost two-thirds of chemicals that were on the AP&VMA National Review of Agricultural Chemicals Program list were not regularly monitored in the environment, and monitoring of the remainder is restricted to high-use areas, such as the cotton growing regions (AP&VMA, 2008). The collection of monitoring data by which the effectiveness of pesticide controls can be assessed tends to be rather *ad hoc* and are not generally designed, collated or used to enable a statistically valid analysis of any chemical management practices (Kookana *et al.*, 1998; DE&H, 2001; Fishel, 2006). Monitoring is a crucial step in understanding the environmental impact of chemicals and for taking subsequent action to reduce the impacts. The most consistent and comprehensive data set for pesticide use anywhere in Australia is the *Central and North-West Regions Water Quality Program*, which has been monitoring five catchments in the cotton growing areas of the north west of New South Wales (NSW) between 1991 - 2010 (NSW Department of Environment, Climate Change and Water

(DECC&W), 2011). Even this program is limited by its weekly (at best) sampling of water column concentrations, and limited sediment monitoring.

Most of the pesticide monitoring data in the Great Barrier Reef region is more than 5 years old (DE&H, 2006), and as stated in the 2001 and reiterated in the 2006 "State of the Environment" (SOE) report by the Department of the Environment and Heritage (Australian Government), it is recommended that more recent information should be collected on the distribution and impact of contaminants in the Reef environment. Currently, pesticide data is collected from the 26 river catchments that drain into the Great Barrier Reef with little or no data taken from the reef itself (DE&H, 2006). It has been identified recently (2006 SOE report) that the threat from persistent pesticides needs to be investigated more thoroughly, and the Australian Government Great Barrier Reef Marine Park Authority (Great Barrier Reef Marine Park Authority, 2008) has begun to investigate the effects of contaminants (pesticides included) on the reef ecosystem; this has been the focus of researchers (Shaw *et al.*, 2009; Shaw *et al.*, 2010).

In the 2001 SOE report, it was highlighted that most State governments were undertaking some form of pesticide monitoring with the most programs in Victoria (21) and NSW (9) (DE&H, 2001; DECC&W, 2011). NHMRC–NRMMC (2004) supported the development of a systematic approach to monitor pesticides (post registration) in groundwaters surrounding the point of application. This monitoring approach has been endorsed world-wide, but is not yet fully integrated into common practice due to the financial costs associated with such a diverse monitoring program. Where groundwater contamination has been detected in Australia, it has usually involved triazine herbicides (see Appendix A). In most of the recent surveys there have been notable reductions in the number of detections now being found (DE&H, 2001; Radcliff, 2002; DECC&W, 2011); however, poor land management practices in a few areas are continuing to create problems with groundwater contamination throughout Australia (Radcliff, 2002; Amis, 2008).

### 2.2.6. Risk to Fauna

Many researchers have investigated the adverse effects of pesticide exposure on wildlife. EXTOXNET (Extension Toxicology Network), the online pesticide database maintained by the University of California, Oregon State University, Michigan State University, Cornell University, and the University of Idaho, provides general chemical, environmental and toxicology information on pesticides. EXTOXNET (1995a; 1995b; 1996a; 1996b; 1996c)

documents atrazine, hexazinone and simazine as being practically nontoxic to wildlife (with exception for aquatic organisms with a >100 mg $kg^{-1}$ lethal dose for hexazinone and simazine). Dicrotophos and monocrotophos are both regarded as very toxic (> 0.19 mg $kg^{-1}$ for avian, aquatic and insect species) and are regarded as one of the most toxic substances to avian populations. As a result, monocrotophos is banned in the USA (registered for use in Australia; APVMA, 2011), and dicrotophos is banned in Australia and across most of Europe (Fry *et al.*, 2001).

Although dicrotophos and monocrotophos are primarily used to control insects in crops, they are best known for their adverse effects on birds. In Texas (1982), dicrotophos laced rice was placed around a rice field to control the birds feeding on the crop; 1,100 birds died over a three week period comprising 12 different bird species (Fry *et al.*, 2001). In the mid-1990s, 20,000 Swainson hawks were found poisoned in their winter grounds in Argentina, dead from monocrotophos exposure (Fry *et al.*, 2001; Stutchbury, 2008). The illegal practice of poisoning bird feed around crops, similar to the incident described in Texas is still widespread, although the extent of mortality on wildlife is not as pronounced. More recently (Stutchbury, 2008), the number of migratory birds were found to be declining in numbers, and pesticides may be a contributing factor. Researchers have discovered that a single application of a highly toxic pesticide to a field can kill 7 to 25 birds per acre; half the birds that are captured after such spraying are found to be suffering from severely depressed neurological function, a common symptom of organophosphate poisoning (Stutchbury, 2008). The USEPA and the American Birds Conservation group are currently lobbying to get dicrotophos banned in the USA (Fry *et al.*, 2001); it is interesting to note that monocrotophos, currently registered for use in Australia and banned in the USA, is a major metabolite of dicrotophos and that dicrotophos is still a commonly used insecticide in many regions throughout the world, in particular on cotton crops in the USA.

As 'harmless' as triazines are believed to be (based on the EXTOXNET assessment), extensive research has been conducted on triazine exposure, with a strong emphasis on the effects of atrazine on wildlife. Figure 2-2 illustrates the range of effects of atrazine, simazine, and hexazinone on fauna conducted by researchers ordered by year published (1996-2009; more detailed information is included in Appendix B for completeness). This selected review excludes research carried out on enzymes and biological markers in isolation.

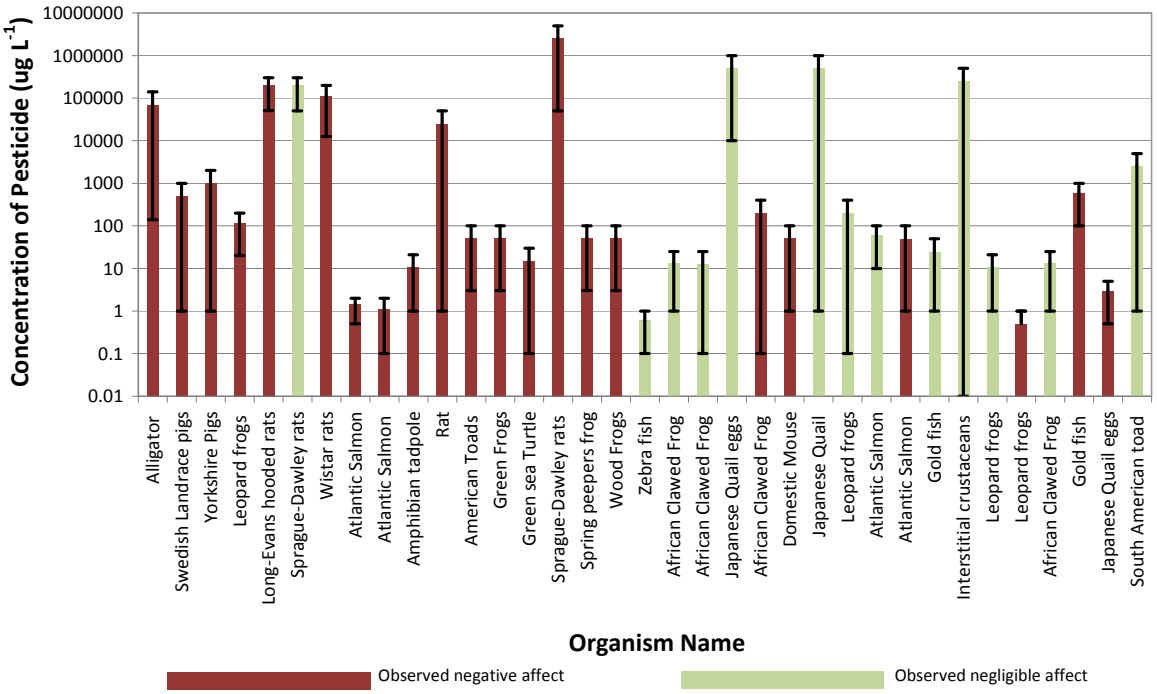



**Figure 2-2: Triazine endocrine disruption and toxicological effects on selected species.**

***Note:*** *organisms are organised by chronological order of publication (publication range from 1996 to 2008). Species tested for atrazine toxicology, n=32. Species tested for simazine toxicology (atlantic salmon and gold fish), n=2. Species tested for hexazinone toxicology (atlantic salmon), n=1. negligible*

As indicated in the data in Figure 2-2, some early researchers concentrated on investigating the effects of large doses of atrazine exposure on a variety of species (Crain, 1997; Cooper *et al.*, 2000; Nadzialek *et al.*, 2008); the results varied but the overall consensus was at low concentrations (typically in the 50-100 μg $L^{-1}$ range) atrazine had no observable effects on growth and development, with an increased incidence of affected growth, development and mortality with higher doses (up to 140 mg $L^{-1}$). Some concluded that pesticide exposure had no significant observable effects on the test species (Allran & Karasov, 2000; Du Preez *et al.*, 2008; Nadzialek *et al.*, 2008). Others recorded significant abnormalities that affected the test species' ability to reproduce after low dose atrazine exposure (0.1 μg $L^{-1}$) (Gojmerac *et al.*, 1999; Moore & Lower, 2001; Hayes *et al.*, 2002; Tavera-Mendoza *et al.*, 2002; Hayes *et al.*, 2003; Keller & McClellan-Green, 2004; Storrs & Kiesecker, 2004; Giusi *et al.*, 2006; Hecker *et al.*, 2006; Brodkin *et al.*, 2007; Fatima *et al.*, 2007; Ottinger *et al.*, 2008). This highlights the complex nature of the determination of the effect of pesticide exposure on wildlife and indicates the complex level of interaction being observed is based on a variety of external conditions: animal life cycle phase, pesticide exposure levels and physical condition of the test species. However, it is worth commenting further on the odd findings of Storrs &

Kiesecker (2004) and Hayes *et al.* (2002 & 2003); Storrs & Kiesecker (2004) found that the survival of animals exposed to lower concentrations of atrazine (3 μg $L^{-1}$) was significantly reduced compared to animals exposed to higher levels of atrazine (100 μg $L^{-1}$) ($p<0.001$ for spring peepers, green frogs and wood frogs). Hayes *et al.* (2002 & 2003) determined that atrazine at low doses (0.1 μg $L^{-1}$) was the reason why male juvenile frogs had observed oocytes (female germ cells) located in the gonads resulting in ovotestis (a gonad with both testicular and ovarian aspects).

Like Storrs & Kiesecker (2004) and Hayes *et al.*, (2002 & 2003) pesticide exposure is discussed by many researchers as one of the factors that cause abnormalities and/or impairment. Sih *et al.* (2004) discusses the concept of pesticides contributing to abnormalities observed in a test species not as being the sole contributor but as a single stressor in an environment that consists of multiple stressors. It is almost impossible to link a single stressor (*i.e.,* a single pesticide, or other organic or an inorganic chemical) to a particular observation in a multiple stressor environment. Research conducted on the African Clawed Frog (*Xenopus laevis)* further illustrates the complexity of pesticide exposure. Hecker *et al.* (2006) flagged atrazine exposure as causing oocytes and ovotestis at low doses, similar to the findings of Hayes *et al.* (2002 & 2003) and Brodkin *et al.* (2007) in Leopard Frogs (*Rana pipien*). Jooste *et al.* (2005) conducted a similar experiment looking only at low dose atrazine exposure (0.1 – 25 μg $L^{-1}$) over a 300-day period. In contrast to Hecker *et al.* (2006), Jooste *et al.* (2005) found that atrazine did not cause oocytes in African Clawed Frogs but instead it was a natural phenomenon observed within frog populations during a specific period in juvenile male frog development. Jooste *et al.* (2005) statistically demonstrated no significant variation between atrazine-exposed frogs and the control group; regardless of exposure, the same percentage of population developed oocytes and ovotestis. This was supported by Coady *et al.* (2005) and taken further by Du Preez *et al.* (2008) who investigated the low dose exposure of atrazine (0.1 – 25 μg $L^{-1}$) on African Clawed Frogs from embryo to adulthood (730 days). Du Preez *et al.* (2008) concluded that atrazine had no effect on reproductive fitness and development of frogs observed. This work further supports Sihs' concept that it is hard to identify a pesticide as the sole contributor to an abnormality or growth and development impairment. Organisms are complex, while laboratory experiments have the ability to investigate pesticide exposure while limiting the number of additional stressors, it should be noted that observed changes are more likely to be a result of a combination of confounding factors that are not considered or out of the

control of the experimental design (*i.e.,* maturity of organisms, metabolomic pathways, species genetics).

In other species, lower levels of atrazine had a more significant effect on wildlife causing reproduction to fail (Japanese Quail), inhibiting enzymes (fish, rats and pigs), and inhibiting growth and development (most species) (see Appendix B for more details). As contradictory as some of the research may be, the underlying conclusion is that pesticides do have a detrimental effect on organisms in the environment; as Sih states, the sum of two stressors are worse than one. With a large proportion of waterways that are monitored showing low levels of pesticides and/or pesticide metabolites, it is of concern that wildlife are being exposed to a variety of 'unnecessary stressors', and it is the accumulation of confounding factors and stressors that are the probable cause for the observed detrimental effect to wildlife.

## 2.3. Pesticide Detection

As discussed in chapter one, water companies apply a risk management framework in order to identify and prioritise contaminants (*e.g.* pesticides, pathogens) that need to be monitored within their drinking water catchments based on likelihood of contamination and consequence of contamination. This is further illustrated when comparing atrazine and hexazinone; both are used widely and have been detected in Australian drinking water catchments. Although hexazinone has a marginally higher probability of leaching into the environment (*e.g.* higher GUS score; *i.e.,* higher likelihood of contamination), atrazine has a significantly lower health guideline value (*i.e.,* much higher consequence of contamination) and is applied in greater quantities resulting in atrazine having a higher priority for monitoring by water companies and government agencies. Establishing a monitoring program based on the risk appraised is a common approach applied; however, it is often undermined by the low frequency of the sampling program (AP&VMA, 2008). With water companies operating within confined budgets and limited resources, monitoring programs are heavily influenced by the associated costs of sampling, sample preparation and the choice of analytical test which can range from AUD100 per sample per analyte (analysis by immunoassay) to AUD580 or more per sample per analyte (for analysis by GC-MS or advanced fluorescence spectroscopy)(2008c). With such high premiums on the analytical analysis of water samples, it is no surprise that pesticide residue monitoring in the Australian environment varies greatly (DE&H, 2001; 2006), with current effective monitoring programs

applied to agriculture intensive areas (*e.g.* where significant large quantities of pesticides applied) under threat of being scaled back (DE&H, 2006; DECC&W, 2011).

### 2.3.1. Current Methods of Detection

The National Environmental Methods Index (NEMI) is a repository of all standard methods for the analysis of a variety of contaminants in various environmental sample matrices, maintained by the Methods and Data Comparability Board, a partnership of water-quality experts from Federal agencies, States, municipalities, industry, and private organisations. The NEMI listed methods described below and tabulated in Table 2.4 are capable of detecting atrazine (and metabolites), simazine, hexazinone, monocrotophos and dicrotophos at very low concentrations (NEMI, 2008).

#### *Gas chromatography with ECD/NPD/MS detection*

The gas chromatography methods (EPA 8141B, 5270D, 505, 507, 508.1, 525.2, 527, 551.1; ASTM D5475; and USGS 0-112-91, 0-1126-95, 0-2002-01, 0-3106-93) all require a pre-filtered sample to be extracted with an organic solvent either by liquid - liquid extraction or solid phase extraction (discussed in more detail in Chapter Seven and Eight). The organic solvent extract is isolated, dried, and concentrated to a known volume (typically 1-5 mL) after adding an internal standard. The concentration of the pesticides in the extract is measured by injecting the extract into a high resolution capillary column gas chromatography (GC) system equipped with either an electron capture detector (ECD), nitrogen-phosphorus detector (NPD) or mass spectrometer detector (MS) for detection, identification, and quantification (NEMI, 2008; USEPA, 2008a). Detection limits range from 0.003 μg $L^{-1}$ (GC-MS with SPE) to 2.4 μg $L^{-1}$ (GC-ECD) for triazines; and 0.005 μg $L^{-1}$ (GC-MS with SPE) to 40 μg $L^{-1}$ (GC-MS) for organophosphates.

#### *High performance liquid chromatography with UV/FL/ESI-MS detection*

The HPLC methods (EPA 526; and USGS 0-2060-01), as per the GC methods, all require extraction of a pre-filtered sample. The organic solvent extract is isolated, dried, and concentrated to a known volume (typically 1 mL). The concentrated pesticides are chromatographically separated by HPLC using either a reverse-phase C-8 or C-18 HPLC column, coupled to either an ultra violet (UV), fluorescence (Fl) or an electrospray ionization interface and quadrupole mass spectrometer (EI-MS) for detection, identification, and quantification (NEMI, 2008; USEPA, 2008a). Detection limits range from 0.005 μg $L^{-1}$ (HPLC-MS with SPE) to 0.04 μg $L^{-1}$ (HPLC-EIS-MS) for triazines.

### *Immunoassay Methods*

Atrazine and simazine can be detected using a colorimetric immunoassay (IA) method (Abraxis, 500001, 500007, 520005; SDI, A00071, A00151, A00246; and Syngenta, AG-625). The methods require a small water sample and an enzyme conjugate solution (enzyme-labeled with pesticide) to be mixed together followed by the addition of paramagnetic particles with attached pesticide-specific antibodies. The pesticide in the sample and the enzyme conjugate compete for antibody binding sites on the paramagnetic particles in proportion to their concentrations. At the end of an incubation period, the magnetic particles are separated (magnetic separator) and washed. A substrate is then added which is catalyzed by the enzyme and converted from a colorless to a colored solution. The reaction is terminated with the addition of a dilute acid. The concentration of pesticide in the sample is determined by measuring its absorbance at a specific wavelength using a spectrophotometer, and comparing its absorbance against a calibration standard. Immunoassays can produce false positives by "like" compounds binding with the antibodies and producing a measured response (NEMI, 2008; Huang *et al.*, 2008). Detection limits range from 0.01 µg $L^{-1}$ to 0.05 µg $L^{-1}$ for triazines.

Many researchers have utilized the inhibitory effects of pesticides on acetyl cholinesterase or other enzymes to develop biosensors for their determination (Wortberg *et al.*, 1993; Ayyagari *et al.*, 1995; Baumner & Schmid, 1998; Marquette & Blum, 1998; Aboul-Enein *et al.*, 2000; Marquette & Blum, 2000; Fähnrich *et al.*, 2001; Jain *et al.*, 2004; Ciumasu *et al.*, 2005; NEMI, 2008). While these methods are sensitive (eg. detection of atrazine as low as 0.03 µg $L^{-1}$; 2,4-D at 0.2 µg $L^{-1}$; TNT at 0.1 µg $L^{-1}$, and diuron at 0.2 µg $L^{-1}$) and relatively cheap in comparison to traditional analytical chromatographic methods, they are considered time consuming and expensive to develop in comparison to conventional FIA systems that utilise chemical or spectrometric detection, they have a limited lifespan, and are required to be operated under temperature controlled laboratory conditions.

### *Fluorescence*

Rodriguez Jr *et al.* (2002) developed a fluorescence method for the determination of atrazine involving a tissue-based biosensor system that uses naturally occurring aquatic photosynthetic tissue. The biosensor only works by incorporating the principles of fluorescence induction in living photosynthetic tissue and monitoring the change in fluorescence when exposed to pollutants. A small sample is introduced to the biosensor,

after an incubation period (30 min) the fluoresence of the aquatic photosynthetic tissue is measured and compared to a blank and standards (Rodriguez Jr *et al.*, 2002; NEMI, 2008). The fluorescent method developed by Rodriguez *et al.* and approved by NEMI for the determination of atrazine in water, including analytical performance of the previous GC, HPLC and immunoassay methods are summarised in Table 2-4. Detection limits were not reported (Rodriguez Jr *et al.*, 2002; NEMI, 2008).

**Table 2-4: Analytical methods for the determination of the selected triazines and organophosphates in water samples.**

| Method Numbers | Analyte | Method Title | Detection Level (µg $L^{-1}$) | Detection level type | Recovery | Precision | Instrumentation | Source |
|---|---|---|---|---|---|---|---|---|
| 8141B (by GC-FPD) | Organophosphate | Organophosphorus Compounds in Water, Soil, and Waste Samples by GC-FPD | N/A | MDL# | - | - | GC-FPD | EPA (USEPA, 2008a) |
| ORNL-01 | Triazine | Toxins in Water by Chlorophyll Fluorescence | N/A | EDL' | N/A | N/A | FLUOR | ORNL from NEMI (2008) |
| O-2002-01 | Triazine | Pesticides and Selected Degradates in Water by C-18 Solid-Phase Extraction and GC/MS | 0.002 | MDL# | 31.6 % | | GC-MS | USGS-NWQL from NEMI (2008) |
| O-2002-01 | Organophosphate | Pesticides and Selected Degradates in Water by C-18 Solid-Phase Extraction and GC/MS | 0.002 | MDL# | 31.6 % | 0.002 ug/L | GC-MS | USGS-NWQL from NEMI (2008) |
| 508.1 | Triazine | Chlorinated Pesticides, Herbicides, and Organohalides in Water by GCECD | 0.003 | MDL# | 140 % | 7.14 % RSD | GC-ECD | EPA (USEPA, 2008a) |
| O-2060-01 | Triazine | Pesticides in Water by SPE and HPLC-MS | 0.005 | MDL# | 74.4 % | 6 % RSD | HPLC | USGS-NWQL from NEMI (2008c) |
| 500007 | Triazine | Atrazine by Immunoassay, High Sensitivity, Magnetic Particle | 0.01 | LOD* | 103 % | 10 % RSD | IA | Abraxis (2008) |
| 507 | Triazine | Pesticides in Water Using GCNPD | 0.02 | MDL# | 101 % | 4 % RSD | GC-NPD | EPA (USEPA, 2008a) |
| A00151 | Triazine | Atrazine in water by Immunoassay, High Sensitivity | 0.02 | MDC** | 108 % | 7.1 % RSD | IA | SDI from NEMI (2008)(2008c) |
| O-1126-95 | Triazine | Pesticides in Water by C-18 Solid-Phase Extraction and GC-MS | 0.02 | MDL# | 89 % | 6 % RSD | GC-MS | USGS-NWQL from NEMI (2008) |
| A00246 | Triazine | Simazine in water by Immunoassay | 0.03 | MDC** | 90 % | 2.6 % RSD | IA | SDI from NEMI (2008) |
| 520005 | Triazine | Atrazine by Immunoassay, Microtiter Plate | 0.03 | LOD* | 104 % | 15 % RSD | IA | Abraxis (Abraxis, 2008) |
| 527 | Triazine | Pesticides and flame retardants in water by SPE and capillary column GC/MS | 0.04 | MDL# | 109 % | 13 % RSD | GC-MS | EPA (USEPA, 2008a) |

| Method Numbers | Analyte | Method Title | Detection Level (µg $L^{-1}$) | Detection level type | Recovery | Precision | Instrumentation | Source |
|---|---|---|---|---|---|---|---|---|
| 536 | Triazine | Determination of Triazine Pesticides and Their Degradates in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS) PDF | 0.04 | MDL# | 50 – 150% | | HPLC EIS-MS | EPA (USEPA, 2008a) |
| A00071 | Triazine | Atrazine in water by Immunoassay | 0.05 | MDL# | 93 % | 5 % RSD | IA | SDI from NEMI (2008) |
| 500001 | Triazine | Atrazine by Immunoassay, Magnetic Particle | 0.05 | LOD* | 106 % | 8 % RSD | IA | Abraxis (Abraxis, 2008) |
| AG-625 | Triazine | Atrazine by Immunoassay | 0.05 | MDL# | 108.7 % | 11.3 % RSD | IA | Syngenta (2008c) |
| 525.2 | Triazine | Organics in Water Using GCMS | 0.08 | MDL# | 109 % | 4.8 % RSD | GC-MS | EPA (USEPA, 2008a) |
| 551.1 | Triazine | Chlorinated Compounds in Water Using GC-ECD | 0.08 | MDL# | 121 % | 3.56 % RSD | GC-ECD | EPA (USEPA, 2008a) |
| O-1121-91 | Triazine | Organonitrogen Herbicides in Water by Solid Phase Extraction, GC/MS | 0.09 | MDL# | 67 % | 18 % RSD | GC-MS | USGS-NWQL from NEMI (2008) |
| O-3106-93 | Triazine | Triazines in Water by Gas Chromatography | 0.1 | MDL# | 90 % | 2.3 % RSD | GC-NPD | USGS-NWQL from NEMI (2008) |
| D5475 | Triazine | Nitrogen and Phosphorus-Containing Pesticides | 0.1 | EDL' | 92.9 % | 14.5 % RSD | GC-NPD | ASTM from NEMI (2008) |
| 505 | Triazine | Pesticides and PCBs in Water GC-ECD | 2.4 | MDL# | 85 % | 16.2 % RSD | GC-ECD | EPA (USEPA, 2008a) |
| 8270D | Organophosphate | Semi volatile Organic Compounds by GC/MS | 40 | EQL+ | - | - | GC-MS | EPA (USEPA, 2008a) |

***NOTE:*** *Analytical methods are organised by method detection limits (smallest to largest). LOD* - Lower limit of detection (3 x standard deviation (SD)) (Tebbutt, 1998); MDL# - Method detection limit. SD of the lowest concentration measureable (n = >7) multiplied by the one-sided t distribution (Tebbutt, 1998); MDC** - Minimum detectable concentration (NEMI, 2008c); EDL' – Estimated detection limit. Estimation calculation is based on the noise ratio, area ratio and height ratio of the analyte peak and internal standard for specific analytes (NEMI, 2008c); and EQL+ - Estimated quantification limit (NEMI, 2008).*

As illustrated in Table 2-4, SPE GC-MS offers the lowest detection limit for both triazines and organophosphates with very high precision and recovery. This indicates that the SPE procedure employed (C18 SPE cartridges) is suitable in removing any possible interfering species that may be present in natural water samples while achieving the required sensitivity of target analytes. HPLC-MS or HPLC-EIS-MS methods are comparable to GC-MS, with the main point of difference being the mode of delivery of sample injection (*i.e.,* GC methods introduce the sample into a gas phase, while HPLC operates within the aqueous phase). However, both GC-MS and HPLC-MS methods require specialised instrumentation which is expensive and they are also relatively expensive methods to set up and maintain (*e.g.* taking into account the price of consumables); this is reflected in the relatively high cost of analysing water samples by these methods, as previously described in section 2.4. Interestingly, less expensive methods (*e.g.* HPLC-UV-Vis and to a greater extent immunoassays) are cheaper in comparison but offer less sensitivity in regard to detection limits, precision and recovery. Immunoassays and conventional HPLC-UV-vis also suffer from potential interfering compounds. It is because of the high premiums associated with more sophisticated methods such as GC-MS and HPLC-MS, and the low reliability in terms of precision and recovery of immunoassays and conventional HPLC that there is a niche requirement to develop a more robust, low cost (in terms of instrumentation and analysis) method for the detection of these pesticides.

### 2.3.2. An Alternative Approach

Although the current methods previously described are able to detect target analytes at concentrations below drinking water guidelines, the costs or limitations of these methods are significant (NEMI, 2008). An alternative is to use FIA. FIA has simple instrumentation, good sensitivity for the detection of a wide variety of analytes and requires little or no pre treatment prior to analysis; however, if sample pre treatment/sample manipulation is required, it can be achieved by adding a simple in-line extraction manifold prior to analysis.

#### *Flow injection analysis (FIA)*

FIA has been described as an automated chemical analysis; it was developed in response to the need to automate chemical analysis for researchers where traditional batch experiments were performed continuously, without variation (Valcárcel & Luque de Castro, 1987). The primary difference between FIA and other robotic approaches is the ability to react to feedback and tailor the instrumentation to the researcher's needs. In its simplest form,

Ruzicka & Hansen (1988) defined FIA as '*an injection of a liquid sample into a moving, non-segmented continuous carrier stream of suitable liquid. The injected sample forms a zone, which is then transported toward a detector that continuously records the absorbance, electrode potential, or other physical parameter as it continuously changes due to the passage of the sample through the flow cell*'.

Although FIA wasn't fully established and reported until the 1970s by Nagy *et al.* (1970), the concepts and theory behind FIA were already recognised (Taylor, 1953 &, 1954; Klinkenberg & Sjenitzer, 1956; Levenspiel & Smith, 1957; Levenspiel, 1958; Levenspiel & Turner, 1970; Lane & Sirs, 1974). Taylor (1953) investigated the effects of soluble solutions in water flowing through cylindrical tubes of various composition, lengths and diameter (*i.e.,* investigation into dispersion effects). Klinkenberg & Sjenitzer (1956) further investigated the effects of dispersion and flow velocity. Levenspiel & Smith (1957) and Levenspiel (1958) studied the effects of longitudinal mixing within flowing streams in cylindrical pipes. Levenspiel & Turner (1970) studied the effects of solution mixing, different sample injection techniques and their effect on mixing. Lane & Sirs (1974) investigated the variability of laminar flow and dispersion.

The system Nagy *et al.* (1970) developed measured the potential of a continuous flowing stream after an electrolyte was injected. At constant potential, the peak area of the voltammetric signal was proportional to the amount of electrolyte introduced. Ruzicka & Hansen (1975) further developed the concept of FIA to produce the type of instrument that is still in use today. It was later developed for the determination of nitrate in environmental waters using an ion-selective electrode (Hansen *et al.*, 1977) and nitrate, potassium, phosphate and ammonia in fertiliser and soil extracts by a potentiometric sensor (an air-gap electrode used in a flow-through unit) and a spectrophotometric arrangement with a flow-through cell (Figure 2-3) (Hansen *et al.*, 1977b).

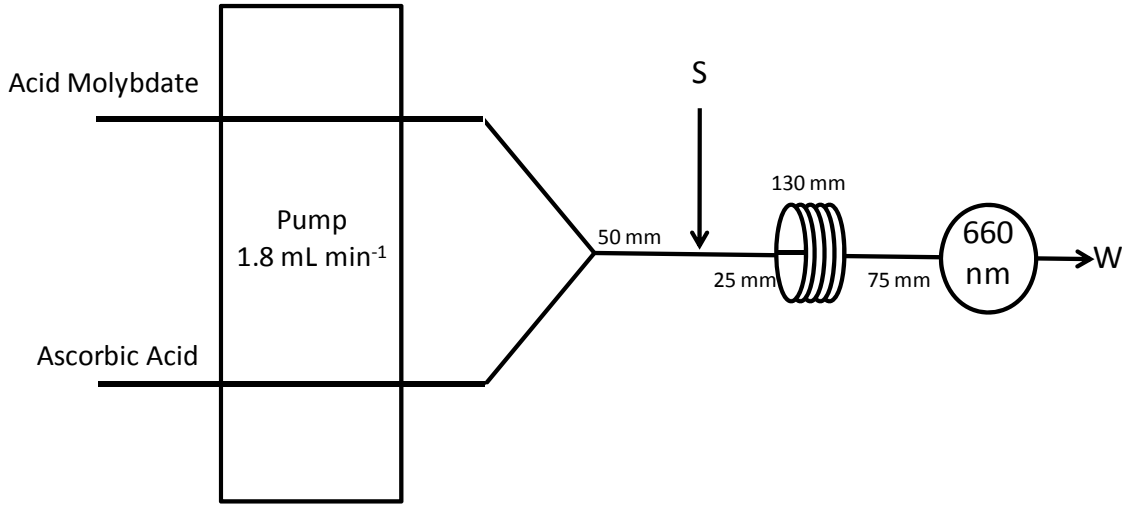



**Figure 2-3: FIA instrument set up for the determination of phosphate in plant material**

***NOTE:*** *FIA instrument developed for the determination of phosphate in plant material. Molybdate and ascorbic acid are pumped and merge to form a carrier stream into which the sample (0.50 mL) is injected. The carrier stream and sample mix in the mixing coil prior to the spectrometric absorbance (660 nm) in which molybdenum blue is measured; S – sample and W – waste, (Ruzicka & Hansen, 1975).*

The system developed by Ruzicka & Hansen (1975) was unique compared to other flow through instruments; it was capable of analysing 200 samples per hour with good resolution (<1% RSD).

FIA has been used for numerous applications for the determination of a variety of water specific and non-water parameters, inorganic and organic compounds in various applications: environmental (inland waters, tap water, sea water, wastes, sediments, air and aerosols (via diffusion into a liquid phase)); food (fruit juices and soft drinks, milk and dairy products, wine and other food products); biological (plants and animal tissues); mineral materials (soil, ores, minerals, ceramics, fertilizers, alloys); clinical (serum, plasma, whole blood and urine); and pharmaceutical and biotechnological applications (Valcárcel & Luque de Castro, 1987; Trojanowicz, 2000).

FIA is a selective analytical method for the determination of a specific analyte in solution. In general, it is the specific analyte-reagent-detection capability of FIA, along with its high reproducibility (<RSD) and high degree of sensitivity which has made FIA widely used. Karlberg & Pacey (1998) list five main factors that contribute to the reproducibility of FIA:

1. Sample/reagent injection volume is fixed.
2. Instrumentation can easily be deconstructed and reconstructed.
3. Flow rate of carrier and sample/reagent can easily be reproduced and controlled.

4. Sample and reagents can easily be adjusted to specific pH with the use of buffers.

5. Simple methods of detection (*e.g.* fluorescence, UV, chemiluminescence etc.).

FIA has many aspects that can be altered in order to obtain better results, such as: flow rate, temperature, reagent type, analyte and reagent pH. Another variation is to alternate the reagent and sample carrier streams (Reverse-phase FIA) (Chantiwas, 2001). Injecting the reagent instead of the sample will reduce the cost of the experiment without altering results; this can only be done when the sample is abundant.

Regular monitoring programs are generally time-consuming and expensive because they require the analysis of hundreds of samples, most of which have pesticide residues below detectable limits. Hence there is a need for a user friendly sensitive screening method which is capable of rapid analyses of multiple analytes, in order to detect pollution events of the type described in Table 2-3, should they occur. This initial screening will reduce the time wasted in analysing hundreds of non contaminated samples by conventional methods. This goal is becoming easier to achieve as screening methods are being developed based on fluorescence, immunoenzymatic and chemiluminescence flow injection techniques.

### *Pesticide determination by flow injection analysis*

Pesticide analysis using flow injection techniques was first reported in 1988 (Mendez *et al.*, 1988). Mendez *et al.* (1988) used a glassy carbon amperometric detector in a flow system for the determination of fenthion (41.7 μg $L^{-1}$) and fenitrothion (140 μg $L^{-1}$) in a methanolic acetate-buffered carrier stream. Since then, numerous researchers have used FIA in combination with various detection methods for the analysis of a variety of pesticides. Pérez-Ruiz *et al.* (1996) investigated FIA fluorescent detection of nabam (0.2 mg $L^{-1}$) and metham (0.9 mg $L^{-1}$) by oxidising thallium (III) to fluorescent thallium (I). Ferré *et al.* (1997) simultaneously determined carbanyl (80 μg $L^{-1}$), carbofurane (1.04 mg $L^{-1}$), propoxur (0.75 mg $L^{-1}$), and isocarb (0.71 mg $L^{-1}$) in water using a multicomponent spectrometric FIA system.

Shi & Stein (1996) developed a flow injection acetyl cholinesterase inhibition immobilized polymer for the determination of paraoxon. The enzymatic reaction was measured using a UV spectrophotometer after a 30 minute analyte-enzyme calibration with a detection limit of 0.05 μg $L^{-1}$. Suwansa-ard *et al.* (2005) developed a semi-disposable stop flow enzymatic biosensor reactor for the determination of carbamate (0.3 mg $L^{-1}$) in water. Acetyl

cholinesterase enzymes were immobilised in silica gel within the biosensor reactor and the reactor was then filled with sample. After 5 minutes incubation any carbamate present would bind with the enzymes. The enzyme inhibition caused by carbamate was measured by pH and conductivity electrodes. Solná *et al.* (2005) further exploited the capabilities of enzymatic FI and developed a multi-enzymatic electrochemical array for the determination of multiple pesticides and phenols. The instrument consisted of tyrosinase, peroxidase, acetylcholinesterase and butylcholinesterase enzyme coated electrodes but was found to perform better in a stopped flow arrangement, with detection limits ranging from 1.7 to 130 $\mu g\ L^{-1}$.

Galeano Diaz *et al.* (1999) incorporated solid phase extraction for the rapid pre-concentration (under 5 minutes) of naptalam with fluorescence detection, reaching a detection limit of 0.03 $\mu g\ L^{-1}$, while Coly & Aaron (1999), and Maniasso *et al.* (1999) used photochemically induced fluorescence for the detection of sulfonylurea herbicides (0.1 $\mu g\ L^{-1}$) and fenvalerate (17 $\mu g\ L^{-1}$). In 2001, Vilchez *et al.* (2001) used the same detection method for imidacloprid (0.3 $\mu g\ L^{-1}$), and Icardo *et al.* (2003) also used it for the detection of sulfonamides (30 $\mu g\ L^{-1}$). García Reyes *et al.* (2003) developed a novel multi-component flow injection system for the simultaneous determination of benomyl (3.0 $\mu g\ L^{-1}$) and carbendazim (7.5 $\mu g\ L^{-1}$) incorporating an in-line pre-concentration gel surface and fluorescence detection flow cell. Simultaneous determination is achieved because of the difference in native fluorescence of the two pesticides. Abaza (1999) and Quintás *et al.* (2004) coupled a fourier transform-raman spectrometer with a continuous FI system for the determination of malathion and chlorsulfuron, fenoxtcarb, folpet, metalaxyl, malathion, primicarb, endosulfuron, fluometron, imidacloprid and buprofezin in pesticide formulations, respectively, with excellent correlation with the traditional GC-FID method. Salinas-Castillo *et al.* (2004) simultaneously measured the phosphorescence of naptalam (8.1 $\mu g\ L^{-1}$) and 1-naphthylamine (a naptalam metabolite)(11.1 $\mu g\ L^{-1}$) with thallium (I) nitrate oxidation.

## Analysis of triazines and organophosphates

The analysis of triazines and organophophates by FIA has been of interest to many researchers. In 1988, Farran *et al.* used an extraction resin (XAD-2) in an unsegmented-flow solid-phase pre-concentration system for the enrichment of organophosphates coupled on-line with a high performance liquid chromatograph with UV detection. Although, the system described is a hybrid FIA-HPLC system, it demonstrated the potential of FIA for pesticide

analysis. In 1992, Farran *et al.* applied the hybrid system to river water samples containing diazinon, azinphosmethyl and fenthion, with detection limits in the order of 70 µg $L^{-1}$. Since then, many researchers have worked to develop analytical methods for the determination of triazines and organophosphates, with a strong focus on atrazine determination. Schobel *et al.* (2000) detailed the research between 1993 and 2000 in the development of pesticide FIA immunoassay methodologies, with atrazine and simazine detection limits between 0.05 to 1.1 µg $L^{-1}$. It was not until 1996 that Martinez *et al.* coupled an in-line stop flow liquid–liquid extraction step for the enrichment of triazines in surface waters into an acceptor stream of n-hexane with spectrophotometric detection (diode array) at 220 – 250 nm. They used a 10 minute pre-concentration step to achieve a detection limit for atrazine of 5 µg $L^{-1}$. Martinez *et al.* (1996) developed one of the first FIA instruments to rival the FIA-HPLC hybrid of Farran *et al.* (1992) that didn't rely on immunoassay detection or enrichment for pesticide analysis.

Table 2-5 lists the FIA methods developed by researchers (before the commencement of this thesis) for the determination of triazines and organophosphates. The table is by no means exhaustive as there are hundreds of publications on immunoassay methods, a large percentage being stop flow FIA methods (not continuous). However, the methods listed do represent the majority of non-immunoassay flow injection methods published.

**Table 2-5: Flow injection methods for the determination of triazines and organophosphates.**

| Year | Analyte | Pesticide Class | Sample Matrix | Instrumentation | Detection Method | LOD (µg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| 1992 | Diazinon, Azinphosmethyl and Fenthion | Organophosphates | River water | FIA-HPLC | UV | 70 | Farran *et al.*, 1992 |
| 1993 | Atrazine | Triazines | N/A | FIA enzymatic immunoassay | Fluorescence | 0.1 | Wortberg *et al.*, 1993 |
| 1993 | Atrazine | Triazines | N/A | FIA enzymatic immunoassay | Fluorescence | 0.1 | Oroszlan *et al.*, 1993 |
| 1994 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Fluorescence | 0.1 | Wittmann & Schmid, 1994 |
| 1994 | Atrazine | Triazines | Water and soil extracts | FIA enzymatic immunoassay | Fluorescence | 1 | in Schobel *et al.*, 2000 |
| 1994 | Organophosphorus and Carbamic | Organophosphates | Water | FIA enzymatic immunoassay | Electrochemistry | <4 | La Rosa *et al.*, 1994 |
| 1995 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Electrochemical | 0.1 | Jiang *et al.*, 1995 |
| 1995 | Paraoxon and Carbaryl | Organophosphates | Water | FIA enzymatic immunoassay | Electrochemistry | 18 | La Rosa *et al.*, 1995 |
| 1996 | Atrazine | Triazines | Drinking water | FIA enzymatic immunoassay | UV | 0.1 | Krämer *et al.*, 1999 |
| 1996 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Electrochemiluminescence | 0.1 | Wilson *et al.*, 1997 |
| 1996 | Atrazine | Triazines | Water | FIA | Reflectometric Interference Spectroscopy | 0.35 | Mouvet *et al.*, 1996 |
| 1996 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | UC | 0.075 | Gascón *et al.*, 1997 |

| Year | Analyte | Pesticide Class | Sample Matrix | Instrumentation | Detection Method | LOD (µg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| 1996 | Atrazine | Triazines | Surface water | FIA | Diode array spectrophotometer | 5 | Martinez *et al.*, 1996 |
| 1996 | Atrazine and Metabolites | Triazines | Water, soil, and liquid food | FIA enzymatic immunoassay | Enzyme inhibition | 0.01-10 | Wittmann, 1996 |
| 1996 | Paraoxon | Organophosphates | Water | FIA enzymatic immunoassay | Enzyme inhibition | 0.05 | Shi & Stein, 1996 |
| 1997 | Simazine | Triazines | Water | FIA enzymatic immunoassay | Waveguide surface plasmon resonance | 0.2 | Mouvet *et al.*, 1997 |
| 1997 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | UV | 0.5 | Bjarnason *et al.*, 1997 |
| 1997 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Fluorescence | 0.02 | Krämer *et al.*, 1997 |
| 1997 | Atrazine | Triazines | N/A | FIA enzymatic immunoassay | Electrochemiluminescence | 10 | Wilson *et al.*, 1997 |
| 1997 | Atrazine and Simazine | Triazines | Liquid phase | FIA enzymatic immunoassay | Total internal reflection fluorescence | N/A | Piehler *et al.*, 1997 |
| 1997 | Dichlorvos and Paraoxon | Organophosphates | Water | FIA enzymatic immunoassay | Electrochemistry | 0.015 | Rippeth *et al.*, 1997 |
| 1997 | Simazine | Triazines | N/A | FIA enzymatic immunoassay | Fluorescence | 0.3 | In Schobel *et al.*, 2000 |
| 1998 | Atrazine | Triazines | Water and soil | FIA | Potentiometric | 0.3 | Hassan *et al.*, 1998 |
| 1998 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Fluorescence | 0.05 | Önnerfjord *et al.*, 1998 |
| 1998 | Atrazine | Triazines | Food | FIA enzymatic immunoassay | Fluorescence | 0.08 | Sendra *et al.*, 1998. |

| Year | Analyte | Pesticide Class | Sample Matrix | Instrumentation | Detection Method | LOD (µg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| 1998 | Triazines | Triazines | Water and urine | FIA enzymatic immunoassay | Fluorescence | 0.5 | In Schobel *et al.*, 2000 |
| 1999 | Atrazine | Triazines | Tap and ground water | FIA enzymatic immunoassay | Fluorescence | 0.7 | Turiel *et al.*, 1999 |
| 1999 | Atrazine | Triazines | Wine | FIA enzymatic immunoassay | Fluorescence | 0.2 | In Schobel *et al.*, 2000 |
| 1999 | Atrazine | Triazines | Ground water | FIA enzymatic immunoassay | Fluorescence | 0.06 | In Schobel *et al.*, 2000 |
| 1999 | Atrazine, Simazine, Deisopropylatrazine, and Deethylatrazine | Triazines | Water | FIA enzymatic immunoassay | Fluorescence | 0.06 and 0.2 | Mallat *et al.*, 1999 |
| 1999 | Organophosphate group | Organophosphates | Potato and cabbage saps | FIA enzymatic immunoassay | Electrochemistry | N/A | Starodub *et al.*, 1999 |
| 1999 | Paraoxon, Chlorpyrifos, and Diazinon | Organophosphates | Tap water and fruit juices | FIA enzymatic immunoassay | Photothermal | 1 - 400 | Pogacnik & Franko, 1999 |
| 1999 | Simazine | Triazines | Ground water | FIA enzymatic immunoassay | Fluorescence | 0.08 | Schobel *et al.* 2000. |
| 1999 | Atrazine | Triazines | Environmental waters | FIA enzymatic immunoassay | Chemiluminescence | 0.03 | Krämer *et al.*, 1999 |
| 2000 | Dichlorvos | Organophosphates | Environmental waters | FIA enzymatic immunoassay | Amperometric | Low ug/L | Neufeld *et al.*, 2000 |
| 2000 | Diclorvos | Organophosphates | Water | FIA enzymatic immunoassay | Fluorescence | 0.5 | Delgado Reyes *et al.*, 2000 |
| 2000 | Atrazine and Simazine | Triazines | N/A | FIA enzymatic immunoassay | Photometric and fluorometric | 0.1 | Fránek *et al.*, 2000 |
| 2001 | Atrazine and Metabolites | Triazines | Water | FIA | MS | 0.1–1.0 | Geerdink *et al.*, 2001 |

| Year | Analyte | Pesticide Class | Sample Matrix | Instrumentation | Detection Method | LOD (µg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| 2001 | Dichlorvos | Organophosphates | Vegetable samples | FIA | Chemiluminescence | 0.8 | Wang *et al.*, 2001 |
| 2002 | Atrazine and Simazine | Triazines | Water | FIA enzymatic immunoassay | Total internal reflection fluorescence | 0.03 | Barzen *et al.*, 2002 |
| 2002 | Atrazine, Simazine, and Prometryn | Triazines | Water | FIA | MS | N/A | Evgenidou & Fytianos, 2002 |
| 2002 | Diazinon | Organophosphates | Environmental waters | FIA enzymatic immunoassay | Potentiometric | 0.07 | Lee *et al.*, 2002 |
| 2003 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Electrochemistry | 0.006 | Yakovleva *et al.*, 2003 |
| 2003 | Monocrotophos | Organophosphates | Water | FIA | Chemiluminescence | 7 | Du *et al.*, 2003 |
| 2003 | Paraoxon, Parathion, Dichlorvos and Diazinon | Organophosphates | Environmental waters | FIA - on a chip | Amperometric/potentiometric | >1000 | Schoning *et al.*, 2003 |
| 2003 | Parathion | Organophosphates | Rice | FIA | Chemiluminescence | 8 | Lu & Xiaoyu Liu, 2003 |
| 2004 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Electrochemistry | 0.024 | Emneus *et al.*, 2004 |
| 2004 | Grotan | Triazines | Metalworking fluids | FIA | MS | 0.1 | Pretty *et al.*, 2004 |
| 2005 | Atrazine | Triazines | Water | FIA enzymatic immunoassay | Chemiluminescence | 0.2 | Ciumasu *et al.*, 2005 |
| 2005 | Dichlorvos, Parathion and Azinphos | Organophosphates | Environmental waters | FIA enzymatic immunoassay | Micro-electrode array | <0.01 | Law & J. Higson, 2005 |
| 2008 | Atrazine | Triazines | Environmental waters | FIA enzymatic immunoassay | Chemiluminescence | 0.003 | Tudorache *et al.*, 2008 |
| 2008 | Atrazine | Triazines | Environmental waters | FIA enzymatic immunoassay | Chemiluminescence | 6.5 | Varsamis *et al.*, 2008 |

| Year | Analyte | Pesticide Class | Sample Matrix | Instrumentation | Detection Method | LOD ($\mu g\ L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| 2009 | Atrazine | Triazines | Environmental waters | FIA bioassay | Biosensor | 216 | Shitanda *et al.*, 2009 |
| 2009 | Dichlorvos and methylparaoxon | Organophosphates | Water | FIA bioassay | Biosensor | 0.001 | Valdes-Ramirez *et al.*, 2009 |

## 2.4. Proposed New Method: FIA Chemiluminescence

The method proposed and described in this thesis is a flow injection chemiluminescence analysis (FICA) method using chemically oxidized chemiluminescent reagents. Chemiluminescence is defined as the production of light as a result of a chemical reaction. During the reaction, one of the reactants forms an excited state intermediate, which then de-excites and emits a small packet of light (Deluca *et al.*, 1985). The emitter could be the product of the reaction, as long as it is not destroyed by the oxidizing agent, but generally it is one of the reactants. The terms "*chemiluminescence*" and "*fluorescence*" at times are interchanged and misinterpreted; both describe the emission of light from a compound returning from an excited state. However, the light is generated differently: in chemiluminescence the emitted light is the by-product of a chemical reaction; in fluorescence the emitted light is a result of a physical excitation of a compound (gamma/UV /Vis radiation) which releases light on returning from its excited state (Deluca *et al.*, 1985).

As illustrated in Table 2-5, researchers have used chemiluminescence in FI-IA methods for more than fifteen years (Schobel *et al.*, 2000). FI-IA involves the binding of the target analyte either directly to a chemiluminescent tagged antibody, or a substrate with detachable chemiluminescent tagged antibodies (Gamiz-Gracia *et al.*, 2005). Luminol is a popular chemiluminescent reagent used to tag antibodies in immunoassay experiments. It is relatively cheap, has a low number of interfering compounds, a wide range of chemical chemilumnescence enhancers (enhancers are chemicals which increase the amount of light emitted) and does not require specialized equipment for detection; luminol can be measured using a fluorescence spectrometer (without excitation), UV spectrophotometer, diode or a photomultiplier module/tube. While there are numerous FIA methods for pesticide detection (as illustrated in Table 2-5), they tend to be analyte specific. It also should be noted, with the advancement in detection methods and sample extraction methods, many of the methods presented are constantly being developed (e.g. new detection methods are being employed that have increased sensitivity, or extraction methods that are more targeted to specific analytes, *i.e.,* immunoassay techniques). However, even though there are advancements in terms of selectivity and sensitivity, there are still recurring issue with many FIA and FI-IA systems that remain the focus for many researchers; these include problems with interfering species (or false readings from 'like'

compounds, such as metabolites or non-target compounds) and overcoming complex sample matrices.

The focus of this research resides in the chemically induced chemiluminescence for the determination of pesticide residues in natural waters. Direct oxidation of chemically produced FICA involves an analyte and a reagent stream being continuously pumped. A reagent/sample mixture is injected into the carrier stream that upon mixing emits light that is recorded by a detector. A typical FICA setup involves a pump, reagent/sample injection port, T-piece connecter, mixing coil, flow cell, photo multiplier, a recorder and several lengths of FIA PEEK (polyaryletheretherketone) tubing (Figure 2-4).

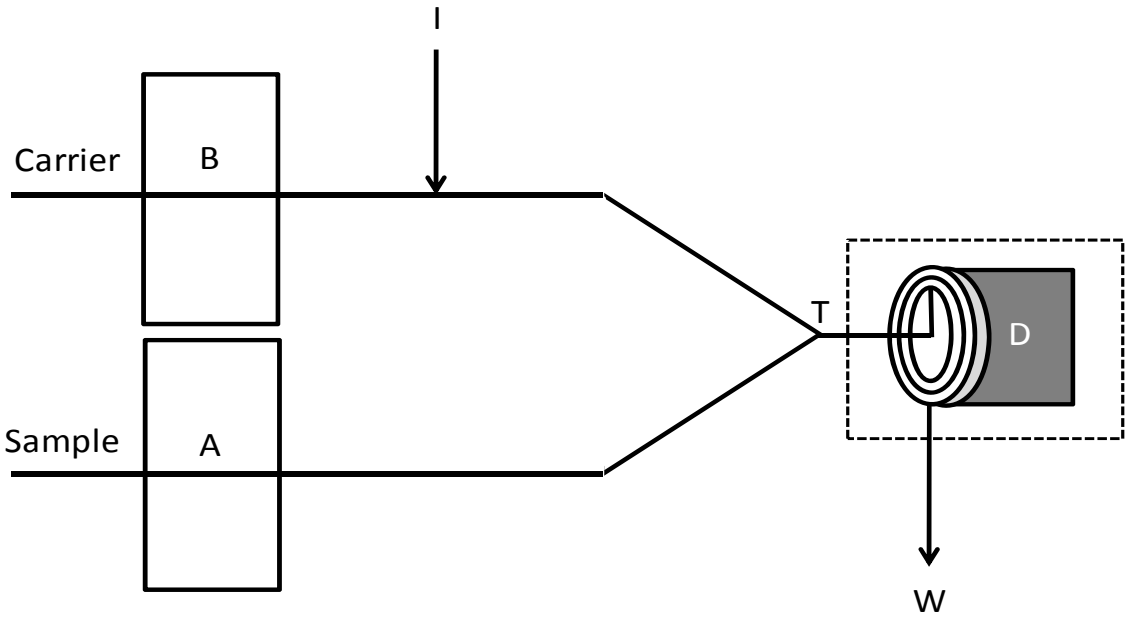



**Figure 2-4: Typical flow injection chemiluminescence analysis schematic.**

***NOTE:*** *(A) Reagent carrier stream peristaltic pump. (B) Sample stream peristaltic pump. (I) Injection valve. (T) T-piece. (D) Photo multiplier tube (PMT). (W) Waste line.*

Chemiluminescence has become increasingly popular (Gamiz-Gracia *et al.*, 2005); it is a cheaper alternative to FI-IA methods, requires less method development and less stringent operating conditions. In addition, FIA with chemiluminescence has been successfully applied to the determination of carbaryl using a variety of chemiluminescent reagents; the detection limits obtained were 4.9 µg $L^{-1}$ with luminol, 29 µg $L^{-1}$ with cerium (IV), 5 µg $L^{-1}$ with peroxyoxalate, and 12 µg $L^{-1}$ using photo generated tris(2,2'-bipyridyl)ruthenium(III) (Pérez-Ruiz *et al.*, 2003; Huertas-Perez *et al.*, 2004; Pulgarin *et al.*, 2006; Tsogas *et al.*, 2009). Wang *et al.* (2001) applied luminol chemiluminescence to the determination of dichlorvos (0.8 µg $L^{-1}$), Du *et al.* (2003) used luminol for the determination of monocrotophos (7 µg $L^{-1}$), and

Adcock *et al.* (2004) used tris(2,2'-bipyridyl)ruthenium(III) for glyphosate determination in commercial formulations.

As demonstrated by Adcock *et al.* (2004) and Perez-Ruiz *et al.* (2003) tris(2,2'-bipyridyl)ruthenium(III) chemiluminesces with aliphatic amines, and as demonstrated by Wang *et al.* (2001) and Du *et al.* (2003) luminol chemiluminesces with organophosphates.

FICA has been demonstrated to be a low cost solution (in terms of its components and operating costs) that is suitable for the determination of pesticide residues in complex samples. In addition, low detection limits have been observed by numerous researchers for a variety of analytes with little or no interferences. Due to FICA having the ability to be analyte specific, be applied to a variety of sample matrices and be robust (in terms of instrumentation and operation), it is a suitable technique that can be investigated for its application as a portable instrument. As such, an objective of this research is to further explore the application and suitability of tris(2,2'-bipyridyl)ruthenium(III) and luminol chemiluminescence in a portable FICA system for the determination of atrazine (and metabolites), simazine, hexazinone, monocrotophos, and dicrotophos.

### 2.4.1. Chemiluminescence of Triazines and Organophosphates

Gerardi *et al.* (1999) and Gorman *et al.* (2006) documented the extensive capabilities of ruthenium as a chemiluminescent reagent. Ruthenium was successfully used to detect a wide variety of analytes, from oxalate and organic acids to amines, in a variety of samples, from synthetic to biological. Costin *et al.* (2003) used tris(2,2'-bipyridyl)ruthenium(III) FICA for the determination of amino acids in the order of 0.5 μg $L^{-1}$. The amino acids selected all consisted of tertiary and secondary aliphatic amines.

#### *Ruthenium*

Based on the work completed and the literature review by Gerardi *et al*. (1999) and Gorman *et al.* (2006) on ruthenium chemiluminescence with amines, it was considered likely that due to the structure of the selected triazines, they would chemiluminesce with ruthenium. Gerardi *et al*. proposed the following reaction mechanism for tris(2,2'-bipyridyl)ruthenium(III) with aliphatic amines (derived from Noffsinger & Danielson, 1987 after Gerardi *et al.,* 1999), Figure 2-5

$Ru(bipy)_3^{2+} \rightarrow Ru(bipy)_3^{3+} + e^-$

$Ru(bipy)_3^{3+} + R'_2NCH_2R \rightarrow Ru(bipy)_3^{2+} + R'_2N^{\bullet+}CH_2R$

$Ru(bipy)_3^{2+} + R'_2N^{\bullet+}CH_2R + H_2O \rightarrow 2H^+ + R'_2NH + OCHR + Ru(bipy)_3^+$

$Ru(bipy)_3^+ + Ru(bipy)_3^{3+} \rightarrow Ru(bipy)_3^{2+} + [Ru(bipy)_3^{2+}]^*$

$[Ru(bipy)_3^{2+}]^* \rightarrow Ru(bipy)_3^{2+} + hv$ (light 610 nm)

**Figure 2-5: Proposed reaction mechanism for tris(2,2'-bipyridyl)ruthenium(III) with aliphatic amines.**

Ruthenium (+3) reacts with the amine to produce a charged amine and ruthenium (+2). The charged amine and ruthenium (+2) react and produce ruthenium (+1). Ruthenium (+1) reacts with ruthenium (+3) to produce an excited ruthenium ([+2]*) and ruthenium (+2). The newly formed excited ruthenium then emits light at 610 nm as the electron falls back down from the excited state. The ruthenium-amine reaction emits light at varying intensities depending on the amine species. A tertiary amine will emit stronger luminescence than a secondary amine. Similarly, the luminescence from the reaction with a secondary amine will be stronger than with a primary amine (Gerardi *et al.*, 1999).

### *Luminol*

Luminol chemiluminescence with organophosphates has been tried with varying success. Wang *et al.* successfully used luminol for the determination of dichlorvos in vegetable samples, but the trials with several other organophosphates (methyl parathion, fenitrothion and malathion) gave very poor responses. Wang *et al.* (2001) investigated the use of various surfactants and their effect on the chemiluminescent intensity emitted. It was found that cetyltrimethyl ammonium bromide (CTMAB) enhanced the signal by 72.5 %. In the work by Du *et al.* (1985) the chemiluminescence from luminol was enhanced with hydrogen peroxide for the determination of monocrotophos. Deluca *et al*. (1985) and Wang *et al.* (2001) reported the proposed luminol reaction mechanisms with organosphosphates, Figure 2-6.

**Figure 2-6: Proposed luminol reaction mechanism with an organophosphate (after Wang *et al.*, 2001).**

Luminol in the presence of a strong base, an organophosphate and hydrogen peroxide, loses the nitrogen protons leaving a negative charge which moves onto the carbonyl oxygen. The oxygen next performs a cyclic addition to the two (previously) carbonyl carbons. Nitrogen is

removed, the charge on the oxygen atoms returns to form carboxylate anions by expelling nitrogen gas. This produces 3-aminophthalate (3-APA*) (in an excited state), which in turn emits light when returning to its ground state (Deluca *et al.*, 1985).

### 2.4.2. Pesticide Chemiluminescence Reaction Sites

All the pesticides selected for this project (with the exception of cyanuric acid, which is an atrazine metabolite) contain tertiary, secondary or primary aliphatic amines. The organophosphate pesticides also have the required phosphate groups that have been proposed to react with luminol. Figure 2-7 (see following page) illustrates the structure of the target pesticides and the proposed reaction sites with either luminol and/or tris(2,2'-bipyridyl)ruthenium(III) chemiluminescent reagents.

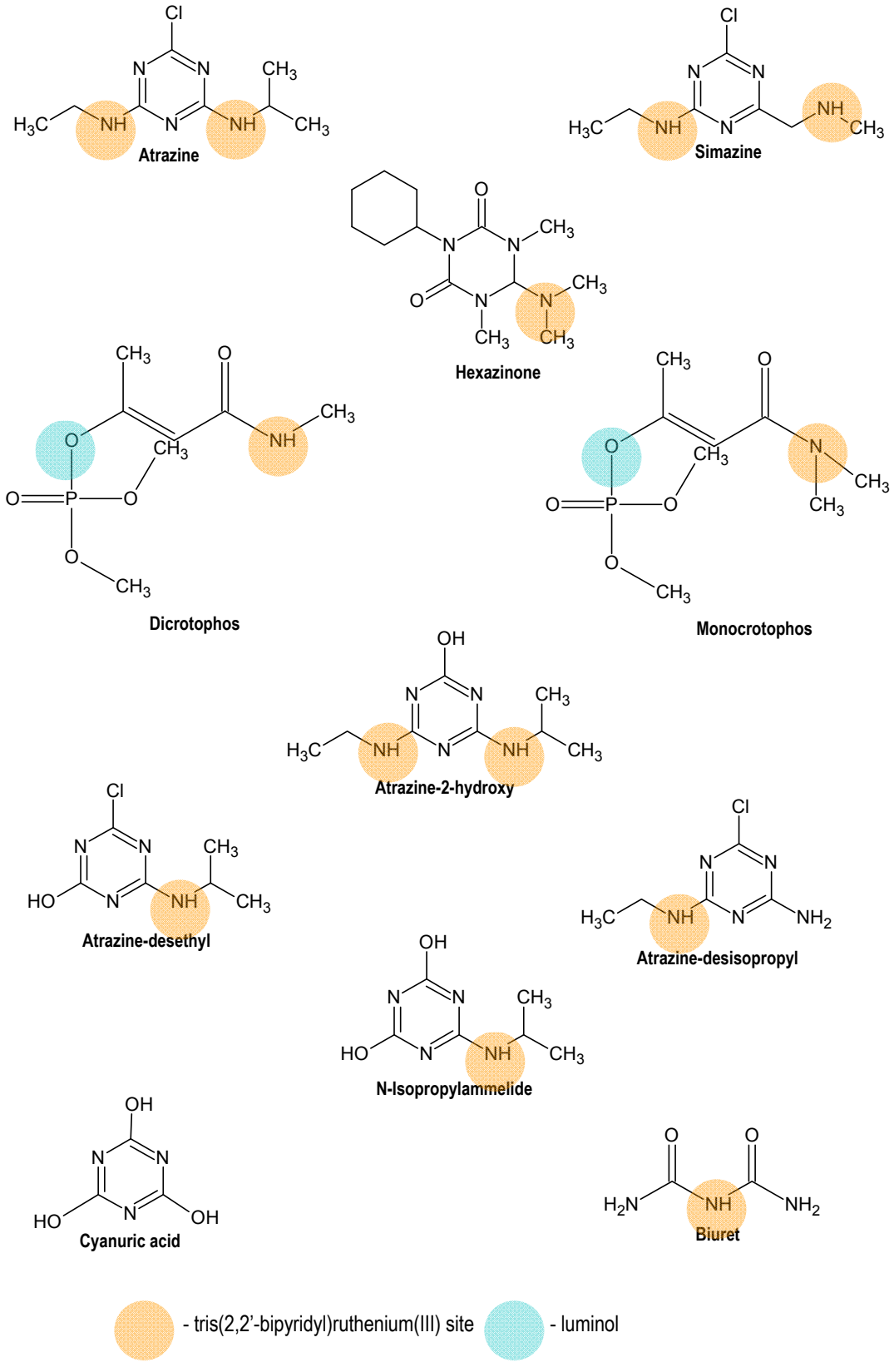



**Figure 2-7: Proposed reaction sites for luminol and tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence for the selected pesticides.**

### 2.4.3. FIA as a Portable Instrument

One feature of all FIA systems is their ability to become portable. This is due to their miniaturization, use of simple, cheap components and their low power requirements. As such, many FIA systems can be developed into analysers that can be placed in-situ or used as portable units. There are already numerous portable FIA systems commercially available, such as the Hach Lange system (Hach Lange Ltd, Manchester, UK) and Systea Micromac (Systea.S.p.A, Via Paduni, IT), both are established systems for the analysis of nutrients (e.g. ammonium, nitrate, phosphate etc.) in aqueous samples. In addition, more advanced research and development units like the FloPro (Global FIA, Seattle, USA) are available for developing more sophisticated methods for specific analytes utilising a variety of detection mechanisms (e.g. chemiluminescence, fluorescence and UV-vis).

It should be noted; the application of many of these commercial FIA systems is limited. Due to their pre-fabrication and limited mode of operation, they are not applicable in all environments or for all analytes (*i.e.*, pesticides). As such, many researchers have adapted FIA methods and developed portable units to suite specific needs and target analytes. For example, Farre *et al.* (2007) developed a portable FIA system utilising a surface plasmon resonance immunosensor for the determination of atrazine in natural waters (LOD 21 ng $L^{-1}$). Mauriz *et al.* (2006a, 2006b, 2006c) developed a similar instrument for the analysis of carbaryl and other organophosphates in natural waters using luminol chemiluminescence detection (>LOD 2.7 µg $L^{-1}$). Turiel *et al.* (1998) developed an immunoassay immobilized on controlled pore glass for the determination of atrazine in natural waters (LOD 2.1 µg $L^{-1}$).

While luminol has been used as a reagent in portable instruments that utilise immunoassay detection methods, tris(2,2'-bipyridyl)ruthenium(III) has not. However, as demonstrated above, the application of tris(2,2'-bipyridyl)ruthenium(III) has been successfully applied to a variety of target analytes with aliphatic amine functional groups in a variety of sample matrices. As such, it is envisioned that tris(2,2'-bipyridyl)ruthenium(III) will be a suitable reagent for use in a portable instrument and is the focus of this investigation.

## 2.5. Research Objectives

The use of tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence for the determination of aliphatic amines has been well documented (*e.g.* see Gerardi *et al.*, 1999); however, the application to environmental samples has been limited (*e.g.* see Perez-Ruiz *et al.*, 2002 &,

2003). The objective of this research is to extend the application of tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence to include environmental samples in a methodology that allows minimal sample preparation in an automated/semi automated flow injection system for the determination of pesticide residues in natural waters. It is envisioned that such a tool would enable water utilities to increase the frequency and rate at which they screen drinking water catchments for pesticide residues.

### 2.5.1. Aims

The aims of this work are four fold, to:

1. investigate the analytical application of tris(2,2'-bipyridyl)ruthenium(III) flow injection chemiluminescence analysis, identifying and overcoming any interfering species commonly found in natural waters
2. establish a reliable and reproducible method for monitoring selected triazine and organophosphate residues in natural waters based on flow injection chemiluminescence analysis
3. detect the selected triazine and organophosphate pesticide residues in water at levels below Australian drinking water guidelines
4. simultaneously analyse and differentiate multiple pesticides and metabolites in natural waters.
5. evaluate the tris(2,2'-bipyridyl)ruthenium(III) chemiclumenescce detection system for its suitability as a portable instrument for the determination of pesticide residue in drinking water

### 2.5.2. Hypothesis

This research aims to investigate the following five hypotheses:

1. tris(2,2'-bipyridyl)ruthenium(III) and luminol chemiluminescence are suitable reagents for the determination of pesticide residues (containing an aliphatic amine or phosphate moiety) in natural water samples
2. natural water samples with varying natural organic matter content will not affect the analytical performance of the proposed instrument

3. the proposed flow injection system is a suitable technological alternative for pesticide screening compared to similar rapid determination methodologies (*i.e.* high performance liquid chromatography)
4. multiple analytes can be detected and differentiated
5. target analytes can be detected below current Australian drinking water guideline values.

# CHAPTER THREE: MATERIALS AND METHODS

This chapter lists the reagents, chemicals and instrumentation used in this study; where warranted, more detailed information on the methods and instruments used are described within the subsequent Chapters.

## 3.1. Reagents and Chemicals

### 3.1.1. Chemiluminescent Reagents

**Tris(2,2'bipyridyl)ruthenium(III)** was prepared by dissolving 74.86 mg of tris(2,2'bipyridyl)ruthenium(II) hexahydrate (obtained from Aldrich, Germany; Analytical reagent (AR)), or synthesised according to Broomhead & Young (1990)) in 100 mL of 0.02 M sulfuric acid (laboratory reagent) with 1.0 g of lead dioxide (Merck, Germany, (AR)). The chemiluminescent reagent was filtered using either a 0.45 µm syringe filter (Nylon, Bonet, Australia) or an in-line filter, constructed in-house from a glass Pasteur pipette and glass wool, prior to reacting with target analyte(s).

**3-Aminophthalhydrazide (Luminol)** was prepared by dissolving 0.2 g of 3-aminophthalhydrazide (Fluka, Switzerland; bio chemiluminescence grade (BG)), 8.0 g of sodium hydrogen carbonate (BDH, England; AR), 11.0 g of disodium carbonate (BDH, England (AR)) in 1.0 L of water (MilliQ water system, Millipore) containing 3.0 mL of 33% hydrogen peroxide. The luminol solution was stored for 24 hours at 4°C in the dark prior to being used to ensure the solution was stabilised.

### 3.1.2. Target Analyte(s)

#### *Triazine herbicides*

**Atrazine stock solution** was prepared by dissolving 100.0 mg of atrazine (Supelco, Germany; Neat analytical standard (NAS)) in 10.0 mL methanol (Riedel-de Haën, Europe; HPLC grade >99.95%). Stock solutions were sonicated for 3 hours, dispersing the atrazine into solution.

**Simazine stock solution** was prepared by dissolving 100.0 mg of simazine (Supelco, USA; NAS) in 10.0 mL methanol (Riedel-de Haën, Europe; HPLC grade >99.95%). Stock solutions were sonicated for 3 hours, dispersing the simazine into solution.

**Hexazinone stock solution** was prepared by dissolving 100.0 mg of hexazinone (Supelco, USA; NAS) in 10.0 mL methanol (Riedel-de Haën, Europe; HPLC grade >99.95%).

### *Organophosphate insecticides*

**Monocrotophos stock solution** was prepared by dissolving 100.0 mg of monocrotophos (PESTANAL®, Riedel-de Haën Europe; NAS) in 10.0 mL methanol (Riedel-de Haën, Europe; HPLC grade >99.95%).

**Dicrotophos stock solution** was prepared by dispersing 122 µL of dicrotophos (Supelco, USA; NAS) in 10.0 mL methanol (Riedel-de Haën, Europe; HPLC grade >99.95%). Note: dicrotophos density equal to 1.216, equivalent mass 100.3 mg.

### *Atrazine metabolites*

**Atrazine-desethyl stock solution**, **atrazine-2-hydroxy stock solution** and **atrazine-desisopropyl stock solution** were each prepared by dissolving 100 mg of atrazine metabolite (PESTANAL®, Riedel-de Haën Europe; NAS) in 10.0 mL methanol (Riedel-de Haën, Europe; HPLC grade >99.95%). Stock solutions were sonicated for 3 hours, dispersing the metabolites into solution.

**Note: All stock solutions were prepared monthly. When not in use the solutions were stored at 4°C in the dark. All working solutions were prepared from stock solutions on the day of analysis.**

A second group of atrazine metabolites as generated using enzymes currently being developed (labelled A-D, confidentiality clause restricts the naming of Orica developed enzymes) by Orica Water Technologies (Australia). The metabolites provided by Orica were atrazine-2-hydroxy (100 mM) generated by enzyme A, N-isopropyl ammelide (100 mM generated with enzyme A and B), cyanuric acid (100 mM) generated with enzyme A, B and C and biuret (100 mM) generated with an enzyme consortium A-D. Samples were stored at 4°C in the dark. All samples provided by Orica were used within 3 months of production. All subsequent biologically produced atrazine metabolites were prepared by adding 20.0 mg of appropriate enzyme or enzyme consortium to a 100 mM atrazine in MilliQ water.

## 3.1.3. Interfering Species

A series of compounds and ions was selected to investigate the effect they have on the determination of the target analyte(s) using chemiluminescence detection. Most of the interfering species used are found in the environment naturally at various concentrations.

Table 3-1 details the interfering species studied, their chemical grade and place of origin. All interfering species were prepared weekly, working solutions were made daily from a stock solution that was stored at 4 °C in the dark when not in use.

**Table 3-1: Interfering species studied in the determination of pesticide residue in water by flow injection chemiluminescence detection.**

| Interfering Specie | Manufacturer | Chemical Grade |
|---|---|---|
| *Cations, Anions and Metals* | | |
| Aluminium sulphate | BDH, England | AR |
| Ammonia | Ajax, Australia | LR |
| Ammonium acetate | Merck, Germany | AR |
| Calcium carbonate | BDH, England | AR |
| Calcium chloride | Merck, Germany | AR |
| Iron sulfate | BDH, England | AR |
| Iron sulfite | BDH, England | AR |
| Magnesium chloride | Ajax, Australia | AR |
| Nickel chloride | Ajax, Australia | AR |
| Potassium chloride | Ajax, Australia | AR |
| Potassium nitrate | BDH, England | AR |
| Sodium bicarbonate | BDH, England | AR |
| Sodium bromide | BDH, England | AR |
| Sodium chloride | BDH, England | AR |
| Sodium nitrite | Ajax, Australia | AR |
| Sodium sulphate | Ajax, Australia | AR |
| Zinc chloride | BDH, England | AR |
| *Amino Acids* | | |
| Alanine | BDH, England | Bio* |
| Glycine | BDH, England | Bio* |
| L-Proline | Supelco, USA | AR |
| L-Tryptophan | BDH, England | Bio* |
| L-Tyrosine | BDH, England | Bio* |
| L-Valine | BDH, England | Bio* |
| *Natural Organic Compounds* | | |
| Fulvic acid | Omnia, Australia | Commercial product |
| Humic acid salt | Aldrich, USA | Technical grade |
| K-Humate | Omnia, Australia | Commercial product |
| Tannic acid | Ajax, Australia | LR |

***NOTE:** *Biological grade chemical, >99.95% purity.*

## 3.2. Laboratory Glassware

Pyroneg (phosphate free, Biological grade, Johnson Diversey, Australia) was used to clean all glassware. A 50 L high-density polyethylene tub was filled with hot water and half a cup of Pyroneg, all glassware was submerged in the Pyroneg solution and soaked for 24 hours. The glassware was then removed, triple rinsed with MilliQ water and let to air dry on a glassware rack prior to use.

## 3.3. Solid Phase Extraction (SPE) Materials

**Bond Elut C18 solid phase extraction cartridge** comprising 40 µm/120 µm irregularly shaped acid-washed silica (0.5 mL cartridge, Varian, USA) were utilised. The Bond Elut C18 cartridge was used with the solid phase extraction manifold kit (Agilent, Vac Elute 12) for manual extractions of target analytes in water samples (see Chapter Seven).

SPE cartridges were pre-conditioned with 5 mL methanol followed by 5 mL MilliQ water prior to sample introduction (1 L aliquot, filtered via 0.45 µm hydrophilic membrane) at 2–4 mL $min^{-1}$ using a 12 port vacuum SPE manifold (Agilent, Australia) and Visiprep SPE tubing (Varian, Australia). The SPE cartridges were then air dried under a vacuum and eluted using 4 mL of 90:10 methyl-tert-butyl-ether:ethyl acetate. Samples were then evaporated to dryness under nitrogen and reconstituted in acetonitrile-MilliQ (30:70 (v/v)), to final eluent volume of 10.0mL.

**Perisorb RP C18 bulk sorbent** (Merck, Germany, HPLC grade) comprising 30/40 µm acid washed silica. The Perisorb was triple rinsed in methanol (Riedel-de Haën, Europe; HPLC grade >99.95%) prior to packing into an in-line solid phase extraction cartridge (170 µL internal volume consisting titanium frits, Global FIA, USA) designed for flow injection analysis (see Chapter Eight).

**Nexus bulk Resin** (Varian, USA) comprising 80/100 µm polymeric sorbent material. The Nexus resin was triple rinsed in methanol (Riedel-de Haën, Europe. HPLC grade >99.95%) prior to packing into a second in line solid phase extraction cartridge (170 µL internal volume consisting titanium frits, Global FIA, USA; see Chapter Eight).

**Magnetic ion exchange DOM resin (MIEX)** (Orica, Australia. Technical grade) comprising 100/300 µm high capacity ion exchange spheres with a magnetized component. The MIEX resin was triple rinsed with 12% NaCl prior to packing into a third in line solid phase

extraction cartridge (170 µL internal volume consisting titanium frits, Global FIA, USA; see Chapter Eight).

## 3.4. Instrumentation

### 3.4.1. Absorbance

Absorbance measurements were conducted on a Cary 300 UV-Vis spectrophotometer (Varian, USA) operating WinUV software (Varian, USA) with quartz cuvettes. All samples were filtered using a 0.45 µm syringe filter (Nylon, Bonet, Australia) prior to analysis. Absorbance was recorded over 190 – 700 nm.

### 3.4.2. Fluorescence

Fluorescence measurements were conducted on a Perkin Elmer LS50B Fluorescence spectrometer (Perkin Elmer, USA) operating FL WinLab (Perkin Elmer, USA) with quartz cells. All samples were filtered using a 0.45 µm syringe filter (Nylon, Bonet, Australia) prior to analysis. The LS50B was primarily used to collect excitation-emission spectra from samples over the range of 200-600 nm (see Chapter Ten).

### 3.4.3. Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Water samples (1 L) were filtered under vacuum (Millipore Amicon 8050, micro/ultrafiltration system) operated under pressure (regulated by N2) at 70 kPa (microfiltration) and 110 kPa (ultrafiltration) prior to the material deposited on the membranes (hydrophilic polyvinylidene fluoride (PVDF) microfiltration membranes, Durapore GVWP, 0.22 µm, Millipore; and hydrophilic polyethersulphone (PES) ultrafiltration membranes, Amicon PBHK, 100 kDa, Millipore) being analysed (while moist) by attenuated total reflectance fourier transform infrared (FTIR) spectroscopy (Perkin-Elmer Spectrum 2000, Perkin Elmer, USA). ATR-FTIR was used for sample natural organic matter identification based on functional groups found in raw water samples (see Chapter seven).

### 3.4.4. High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using a Waters HPLC system (M-6000A, Waters Associates Inc; USA) operated isocratically. Samples were injected via a Waters HPLC injection valve fitted with a 500 µL loop using a 2 mL glass barrel syringe. The HPLC system was connected to a UV-vis detector (SPD-10AV, Shimadzu, Japan) coupled to a personal computer running

Chemstation (Varian, USA). Method specifics (including column details) are described in greater detail in Chapter Five: Optimisation of the HPLC Validation Method. HPLC was primarily used to cross validate the flow injection method(s).

### 3.4.5. Gas Chromatography

Two GC methods were utilised: GC-MS and GC FID. GC-MS analysis was conducted on a Hewlett Packard 6890 while GC-FID utilised a Hewlett Packard 5890 system. The following operating conditions were applied to both methods: Injector temperature 225°C; split valve opened at 0.75 min; split flow 70 mL $min^{-1}$; injection volume 1.0 μL; temperature program: 40°C for 2 min then increasing 20°C $min^{-1}$ to 300°C; Carrier gas (helium) flow: 33 cm $s^{-1}$ at 37°C; Makeup gas (nitrogen) flow: 20 mL $min^{-1}$; Column: BPX5, L 30m, (ID 0.25 mm, Film thickness 0.25 μm). The GC-MS was primarily used to collect sorption isotherm data for solid phase extraction resins and to validate target analyte concentrations in water samples (see Chapters Eight and Nine).

### 3.4.6. Flow Injection Analysis (FIA)

Sample and carrier streams were propelled using a peristaltic pump (Gilson Minipulse, GB) through bridged PVC tubing (1.85 mm i.d; Global FIA, USA). All other tubing was PTFE (0.76 mm i.d; Global FIA, USA). The chemiluminescent reagent was injected into the carrier stream via a six-port injection valve (Rheodyne, USA). Eluent and an optional fourth carrier/reagent solution were propelled using bidirectional syringe pumps (Milligat Global FIA, USA). The reagent and sample streams merged at a T-piece positioned 20 mm from a coiled PTFE (0.5 mm i.d.) flow cell. The flow cell was mounted flush against the window of the red sensitive photomultiplier tube (PMT) (H5784-20 Hamamatsu, Japan GB), which was powered by a stable power supply (see Figure 3-1 Photomultiplier configuration). Output from the PMT was monitored using a chart recorder or recorded through purpose-built software (LabVIEW Vi) using a personal computer. All tubing lengths and composition, as well as instrument configuration schematics are detailed in the appropriate chapters for the specific analyte(s) or analysis.

#### *Instrument control*

LabVIEW (National Instruments, Version 7.1) is a graphical programming environment used to write software for scientific instrumentation. LabVIEW was loaded onto a personal computer operating Windows NT 3.0, and used to write an in-house software application to

operate and regulate flow of the milliGAT pumps, acquire and record data from the photomultiplier via a data acquisition card (National Instruments, USA).

***Photomultiplier configuration***

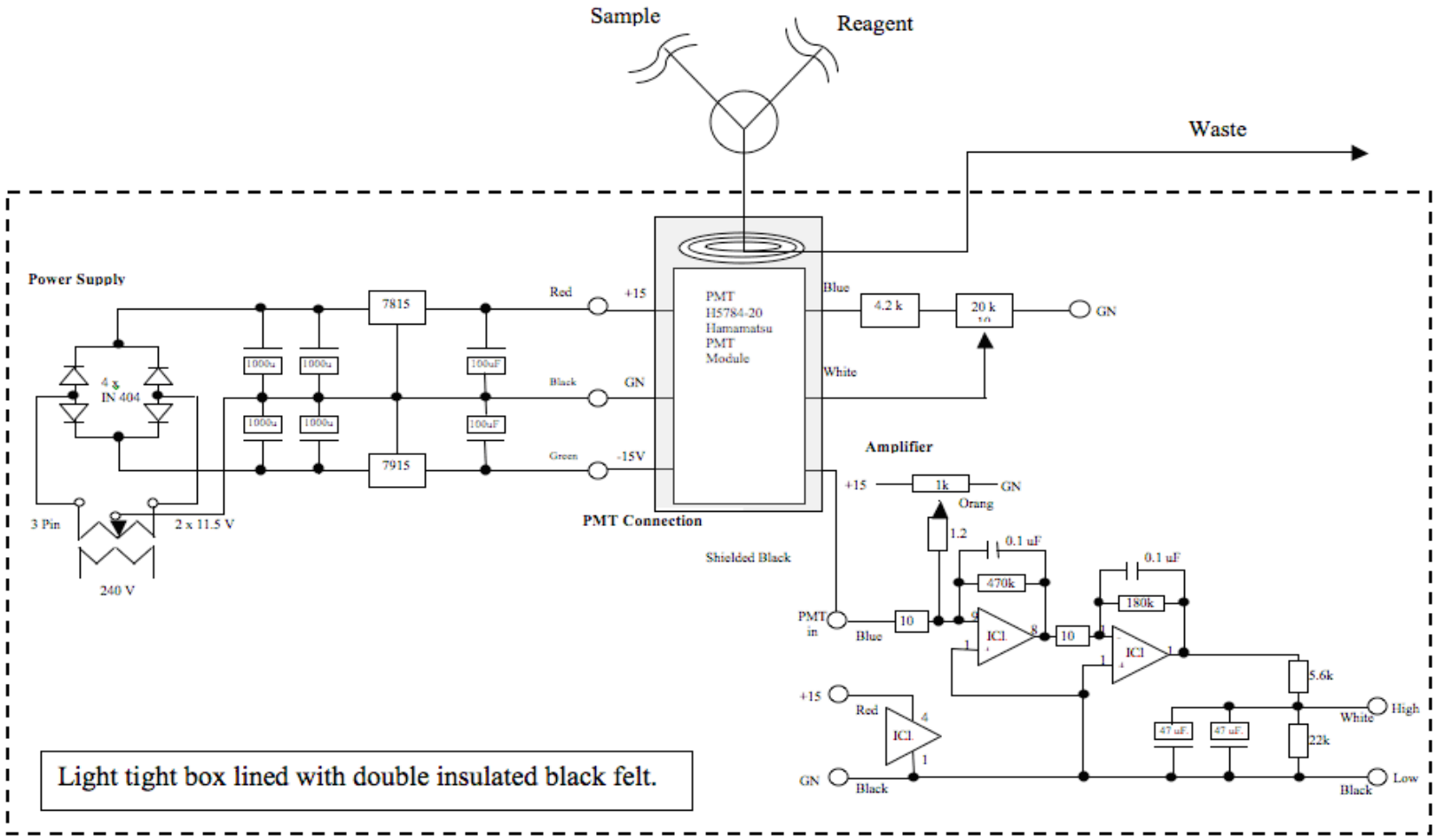



**Figure 3-1: Photomultiplier configuration used in all flow/sequential injection analysis experiments.**

***NOTE:*** *Power supply, amplifier and voltage gain control (for photomultiplier) were all built in house.*

# CHAPTER FOUR: OPTIMISATION OF FIA CHEMILUMINESCENCE PARAMETERS

This chapter outlines the construction and optimisation of the proposed FICA instrument for the determination of pesticide residues in natural waters; first, the key FICA parameters are identified, and through a combination of multivariate and univariate optimisation they were optimised. Second, a series of standards were used to determine the limit of detection. In the succeeding chapters the optimised parameters were applied to natural waters spiked with pesticide(s), along with validation by HPLC and an investigation into possible interfering species.

As previously noted in Chapter Two, many authors have used FIA for the determination of pesticide residues in various sample matrices (*e.g.* natural waters, fruit and plant extracts, commercial waste etc.). However, the processes that were undertaken to optimize the operating conditions of the FI instruments are complex and involve significantly different approaches to method development; some authors applied a univariate optimisation method (Shi & Stein, 1996; Galeano Diaz *et al.*, 1999; Wang *et al.*, 2001; Perez-Ruiz *et al.*, 2002; Townshend *et al.*, 2004), while others applied a multivariate optimisation method (de la Cruz *et al.*, 1995; Ferré *et al.*, 1997; Luna *et al.*, 2000; Salinas-Castillo *et al.*, 2004).

The most common and popular form of FIA optimization cited in the literature is a univariate optimization. Univariate optimization involves varying each parameter in turn, while keeping the other parameters constant until an adequate response is obtained. However, this type of optimization methodology has limitations: firstly, it is not reasonable to assume that each parameter is independent of the other and secondly, the experimental load needed to achieve optimal conditions can be excessive (*i.e.,* the number of experimental runs can be excess of 200)(Luna *et al.*, 2000).

An alternative approach to univariate optimization and an approach that is increasingly becoming more widely applied (although not new) is the use of statistically designed experiments, also known as multivariate optimization. There are three forms of statistical multivariate optimization approaches commonly used, namely: simplex, factorial and central composite (see Table 4-1)(after Luna *et al.*, 2000).

**Table 4-1: Common statistical multivariate optimization approaches (after Luna *et al*, 2000)**

| Optimisation Approach | Advantage | Disadvantage |
|---|---|---|
| **Simplex:**<br><br>A simplex algorithm is applied; where the number of factors (N) determine the complexity of the multidimensional space (N+1) to which linear optimisation occurs between two factors in series. | A maximum number of design points are arranged in a linear uniform manner that covers the range of factors investigated. Additional interior points are included, improving coverage of the overall linear design and enhancing the range of points and variation between the experimental factors. | Often requires a large number of experimental runs (*e.g.* similar to a univariate optimization; magnitude of hundreds) for optimisation designs comprising multiple factors (*i.e.,* multidimensional space; *e.g.* 4 or more). In addition, simplex optimisation approaches can either over shoot or be prematurely stopped before reaching the optimal endpoint. |
| **Factorial:**<br><br>A simultaneous study of the effects from several parameters. As the number of parameters in the factorial design increases, the number of runs necessary to perform the optimization escalates. | A fractional factorial design could be applied in place of a full factorial design to reduce the number of experimental runs. A fraction factorial design uses a subset (either ½ or ¼ fraction of the required experimental runs) of a full factorial design to which information about the main effects and low-order interactions between factors can be inferred.<br><br>The subset is chosen to exploit the 'sparsity-of-effects principle' (i.e., two factor interactions are more common than three and four factor interactions) and as such exposes information about the most important features of the problem investigated, while using a fraction of the effort and resources. | Difficult to apply to situations where multiple factors (*e.g.* three, four or more) interact. |

| Optimisation Approach | Advantage | Disadvantage |
|---|---|---|
| **Central composite:** A factorial or fractional factorial design with centre points, improved with a group of axial (or star) points that allow estimation of curvature. Face centered designs are a type of central composite design with an alpha of 1 (axial points or "star" points are at the centre of each face of the factorial space, between the maximum and minimum variable for each parameter). | Increased confidence in output compared to factorial or fractional factorial design due to the inclusion of star or centre points (demonstrates robustness of design). | Difficult to apply to situations where multiple factors (*e.g.* three, four or more) interact. |

Identifying which FIA parameters to include in an optimisation design is a difficult challenge; it depends on the chemical reaction and the timing of the reaction, *e.g.* de la Cruze *et al.* (1995) and Luna *et al.* (2000) only identified two key factors: carrier flow rate and dispersion coil length in a multivariate optimisation design with confirmation by univariate optimisation. Melquiades *et al.* (2007) identified five factors: sample pH, sample volume, reagent concentration, reagent volume, and stirring time (time before analysis). However, the complexity of the challenge is amplified when identifying parameters in FI systems utilising chemiluminescence (*i.e.,* FICA). In FICA, timing is an additional factor of importance. As such, the detector needs to be as close to the chemiluminescence reaction as possible to ensure the maximum amount of light is captured.

Wang *et al.* (2001) identified flow rate, pH and reagent concentration as the most important factors to include in a univariate optimisation design for organophosphate determination by luminol chemiluminescence; Xu *et al.* (2004) selected reagent pH, reagent concentration, carrier concentration, sample concentration and total flow rate.

Pérez-Ruiz *et al.* (2002) undertook a univariate optimisation design for a four channel, dual injection FICA system for the determination of promecarb and carbofuran by tris(2,2'bipyridyl)ruthenium(III) chemiluminescence. They identified fourteen experimental factors, including the two injection points for the sample, two carrier lines to which the sample was injected, two buffer lines, flow rates of the 4 lines, and the concentration of the two reagents as well as reagent injection volumes. Similarly Townshend *et al.* (2004)

identified ten factors for the determination of tetracycline by tris(2,2'bipyridyl)ruthenium(III) chemiluminescence.

As demonstrated by the literature, the complexity of the optimisation design varies depending on the complexity of the instrumentation utilised and the analysis being performed. In the current optimisation experiment to determine atrazine and other triazines using FICA, the main operational parameters as identified from the literature, were:

- **Flow rate (sample and carrier):** a very important FICA parameter. The ideal flow rate will deliver the sample plug to the detector at the point of maximum chemiluminescence and minimal sample dispersion (*e.g.* Pérez-Ruiz *et al.*, 2002; and Townshend *et al.,* 2004).
- **pH:** as demonstrated by previous authors (*e.g.* Perez-Ruiz *et al.*, 2002 &, 2003; Costin *et al.*, 2004b; Townshend *et al.*, 2004), sample and carrier pH can have a significant influence on the intensity of the chemiluminescence reaction. Also, due to the pH dependence of the chemiluminescent reaction, the reagent pH is most likely to have a similar effect (Pérez-Ruiz *et al.,* 2002).
- **Concentration:** reagent concentration, sample and carrier ionic strength have been demonstrated in the literature to influence the intensity of the FICA response (*e.g.* Pérez-Ruiz *et al.,* 2002 and Townshend *et al.,* 2004).
- **Injection volume:** the amount of reagent injected has been shown to affect the intensity of the chemiluminescence response, as it is independent of the physical characteristics of the FI instrument (*e.g.* Pérez-Ruiz *et al.*, 2002 and Townshend *et al.,* 2004).

The effect of dispersion coils has been identified by numerous authors as an important factor; however, it was not considered for inclusion in the optimisation methods in this research. Previous work by Perez-Ruiz *et al.* (2002) illustrated that dispersion coils would be irrelevant for the chemiluminescent determination of atrazine (and other triazines based on work presented on carbofuran). It was found that the distance required from the point of mixing (at the T-piece) and the detector needed to be kept to a minimum in order to capture the maximum chemiluminescence response. To satisfy this requirement, the flow cell was designed and constructed from a coiled piece of tubing, tightly wrapped and placed flush against the window of the photomultiplier tube window, which acts as both a flow cell and a

dispersion coil to enable reagent and sample mixing. The size of the 'dispersion coil' flow cell was restricted by the size of the photomultiplier tube window. As such, a 310 mm PTFE coiled flow cell (0.5 mm i.d.) was maintained for all experiments.

A multivariate face centered central composite optimization (*i.e.,* a central composite design; see Table 4-1) approach was adopted to study the effects of each parameter chosen (sample and carrier flow rate, sample, carrier and reagent pH) on the chemiluminescence response from tris(2'2-bipyridyl)ruthenium(III) with atrazine, using a set of experiments created from a central composite design. Statistical treatment was performed using MiniTAB 14 software (MiniTAB Inc., USA) to establish the interactions of the selected parameters and their independent effect on the measured chemiluminescence response. To complement this multivariate assessment, univariate studies on the reagent concentration, carrier flow rate, sample flow rate, injection volume and sample pH were also performed. Central composite optimisation was chosen for the optimisation procedure as it finds the optimal levels within the design variables selected by adding a few more experiments to a full factorial design. As such, it establishes a more robust optimisation of each variable compared to a factorial optimisation.

## 4.1. Experimental

### 4.1.1. Solution Preparation

All stock solutions, chemicals and reagents were prepared as described in Chapter Three. A 100 µg $L^{-1}$ aqueous solution of atrazine was used for all optimisation experiments, unless otherwise stated.

### 4.1.2. FIA chemiluminescence Instrumentation

The FIA instrument used for the optimisation and determination of atrazine in water is described in the previous Chapter (section 3.4.6); Figure 4-1 is a schematic of the instrument.

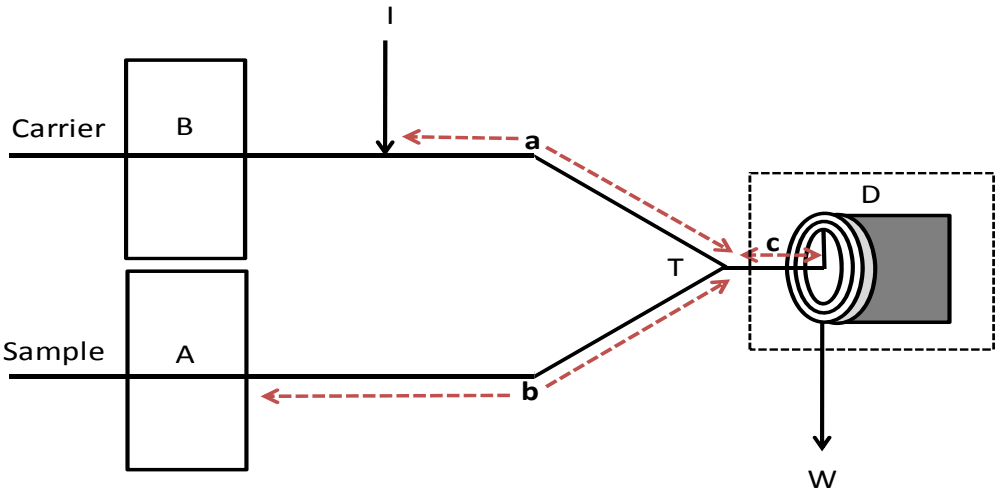



**Figure 4-1: FIA schematic for tris(2'2-bipyridyl)ruthenium(III) chemiluminescence for triazine determination.**

***NOTE:*** *(A) A MilliQ water carrier stream propelled by a peristaltic pump with bridged PVC tubing (1.85mm i.d.; Global FIA, USA). (B) A buffered sample stream (50 mM borax) propelled by a peristaltic pump with bridged PVC tubing (1.85mm i.d.; Global FIA, USA). (I)100 µL tris(2'2-bipyridyl)ruthenium(III) regent via Injection valve. (T) T-piece. (D) PMT. (W) Waste. Tubing lengths (PTFE (0.5mmi.d.; Global, USA)): (a) 11 cm. (b) 20 cm. (c) 2 cm.*

### 4.1.3. Direct Injection HPLC (Validation Method)

HPLC direct injection (Perkins *et al.*, 1999) was carried out with a Waters HPLC system (M-6000A, Waters Associates Inc; USA.) operated isocratically with a water-acetonitrile-methanol (60:25:15, v/v/v) mobile phase at a flow rate of 1.5 mL $min^{-1}$. Aqueous samples were injected (500 µL) via a Waters HPLC injection valve fitted with a 500 µL loop using a 2 mL glass barrel syringe. The injected sample passed through a C8, 5 µm 250 x 4.6 mm column (model 831815 Spherisorb, Phase Separation, USA). The HPLC system was connected to a UV-vis detector (SPD-10AV, Shimadzu, Japan) set at 220 nm coupled to a chart recorder (Model 3395, Hewlett Packard, USA).

### 4.1.4. Optimization of Experimental Parameters

The face centered central composite optimization generated using MiniTAB 14, included 5 parameters (factors) producing a total of 96 experimental runs consisting of 32 cube points, 44 center points and 20 axial points (½ fraction factorial design, in duplicate). Table 4-2 provides the FICA parameters selected for the optimization experiment along with the high (+1), center (0) and low (-1) values. Table 4-3 lists the 96 experiments.

**Table 4-2: Selected FICA parameters for optimization.**

| FICA Parameters | High | Centre | Low |
|---|---|---|---|
| Carrier flow rate mL $min^{-1}$ | 8.0 | 4.5 | 1.0 |
| Sample flow rate mL $min^{-1}$ | 8.0 | 4.5 | 1.0 |
| Sample pH | 12.0 | 9.0 | 6.0 |
| Carrier pH | 8.0 | 4.5 | 1.0 |
| Reagent pH | 4.0 | 2.0 | 1.0 |

The parameter ranges outlined in Table 4-2 were selected for a variety of reasons, namely:

- **Carrier and sample flow rate:** the upper and lower limitations of the pump flow rate were chosen to represent a wide range of possible flow rates that could be used in FICA to optimise the chemiluminescence reaction. While an option of varying the point of mixing and the length to the detector could have been varied, altering the flow rate to capture the maximum signal was undertaken. A maximum of 8 mL $min^{-1}$ was selected in accordance with acceptable operating conditions of the instrumentation (*i.e.,* in regard to the operating pressure).
- **Carrier and sample pH:** the ideal pH for the carrier and sample for tris(2'2-bipyridyl)ruthenium(III) chemiluminescence has been investigated by numerous authors (*e.g.* see Costin *et al.*, 2003; Adcock *et al.*, 2004; Costin *et al.*, 2004a; Gorman *et al.*, 2007). The carrier pH has been shown to be effective in the range from pH 1.0 to 8.0, while the sample pH needs to be between pH 6.0 and 12.0.
- **Reagent pH:** the pH of the reagent was varied in conjunction with the sample and carrier pH to identify the ideal instrumentation pH matrix for the analysis. It is evident in the literature the pH of the analysis is an important factor in chemiluminescence, therefore the reagent pH was investigated over the range of pH 1 to 4. A maximum of pH of 4 was chosen because above pH 4 the reagent becomes unstable, becoming reduced from its oxidised form.

**Table 4-3: Experimental optimization characterisation**

| Run # | FIA Parameter | | | | | Run # (cont…) | FIA Parameter | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | | **A** | **B** | **C** | **D** | **E** |
| 1 | 0 | 0 | 0 | 0 | 0 | 49 | 0 | 0 | 0 | +1 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 0 | 0 | -1 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 51 | +1 | -1 | +1 | -1 | +1 |
| 4 | 0 | 0 | 0 | 0 | 0 | 52 | 0 | 0 | 0 | 0 | 0 |
| 5 | -1 | -1 | +1 | +1 | +1 | 53 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 54 | 0 | 0 | 0 | 0 | 0 |
| 7 | +1 | -1 | +1 | -1 | +1 | 55 | +1 | +1 | +1 | -1 | -1 |
| 8 | 0 | 0 | 0 | 0 | 0 | 56 | +1 | +1 | +1 | -1 | -1 |
| 9 | -1 | +1 | -1 | +1 | +1 | 57 | -1 | +1 | -1 | -1 | -1 |
| 10 | -1 | -1 | +1 | +1 | +1 | 58 | -1 | 0 | 0 | 0 | 0 |
| 11 | 0 | 1 | 0 | 0 | 0 | 59 | -1 | -1 | -1 | -1 | 1 |
| 12 | 0 | 0 | 0 | 0 | 0 | 60 | +1 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 61 | +1 | -1 | -1 | -1 | -1 |
| 14 | 0 | -1 | 0 | 0 | 0 | 62 | +1 | +1 | -1 | -1 | +1 |
| 15 | 0 | 0 | 0 | 0 | 0 | 63 | 0 | 0 | 0 | 0 | 0 |
| 16 | +1 | -1 | -1 | +1 | +1 | 64 | 0 | 0 | 0 | 0 | -1 |
| 17 | -1 | -1 | +1 | -1 | -1 | 65 | +1 | -1 | -1 | +1 | +1 |
| 18 | -1 | +1 | +1 | +1 | -1 | 66 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 67 | -1 | +1 | +1 | -1 | 1 |
| 20 | 0 | 0 | 0 | 0 | 0 | 68 | 0 | 0 | 0 | +1 | 0 |
| 21 | 0 | 0 | 0 | 0 | 0 | 69 | -1 | +1 | -1 | +1 | +1 |
| 22 | 0 | 0 | 0 | 0 | -1 | 70 | 0 | 0 | 0 | 0 | 0 |
| 23 | 0 | 0 | 0 | 0 | 0 | 71 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 72 | 0 | 0 | -1 | 0 | 0 |
| 25 | -1 | -1 | -1 | +1 | -1 | 73 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | 0 | 74 | +1 | +1 | +1 | +1 | +1 |
| 27 | 0 | 0 | 0 | 0 | 0 | 75 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 76 | 0 | 0 | 0 | 0 | 0 |
| 29 | 0 | 0 | 0 | 0 | 0 | 77 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | -1 | 0 | 78 | 0 | 0 | 0 | 0 | 0 |
| 31 | 0 | 0 | 0 | 0 | 0 | 79 | -1 | 0 | 0 | 0 | 0 |
| 32 | -1 | +1 | -1 | -1 | -1 | 80 | -1 | +1 | +1 | -1 | +1 |
| 33 | 0 | 0 | -1 | 0 | 0 | 81 | -1 | +1 | +1 | 1 | -1 |
| 34 | 0 | 0 | 0 | 0 | 0 | 82 | 0 | 0 | 0 | 0 | 0 |
| 35 | +1 | -1 | +1 | +1 | -1 | 83 | +1 | +1 | -1 | -1 | 1 |
| 36 | 0 | 0 | +1 | 0 | 0 | 84 | 0 | 0 | 0 | 0 | 0 |
| 37 | 0 | 0 | 0 | 0 | +1 | 85 | 0 | 0 | 0 | 0 | 0 |
| 38 | 0 | 0 | 0 | 0 | 0 | 86 | 0 | 0 | 0 | 0 | 1 |
| 39 | 0 | 0 | 0 | 0 | 0 | 87 | +1 | -1 | +1 | +1 | -1 |
| 40 | 0 | 0 | 0 | 0 | 0 | 88 | 0 | 0 | 0 | 0 | 0 |
| 41 | +1 | +1 | -1 | +1 | -1 | 89 | -1 | -1 | -1 | -1 | +1 |
| 42 | 0 | -1 | 0 | 0 | 0 | 90 | 0 | +1 | 0 | 0 | 0 |
| 43 | 0 | 0 | 0 | 0 | 0 | 91 | +1 | +1 | -1 | +1 | -1 |
| 44 | 0 | 0 | 0 | 0 | 0 | 92 | 0 | 0 | 0 | 0 | 0 |
| 45 | 0 | 0 | 0 | 0 | 0 | 93 | 0 | 0 | +1 | 0 | 0 |
| 46 | -1 | -1 | -1 | +1 | -1 | 94 | +1 | +1 | +1 | +1 | +1 |
| 47 | 1 | 0 | 0 | 0 | 0 | 95 | -1 | -1 | +1 | -1 | -1 |
| 48 | +1 | -1 | -1 | -1 | -1 | 96 | 0 | 0 | 0 | 0 | 0 |

***Note:*** *A = Carrier flow rate (mL $min^{-1}$), B = Sample flow rate (mL $min^{-1}$), C = Sample pH, D = Reagent pH, E = Reagent concentration (mM), +1 = high point, 0 = center point and -1 = low point.*

## 4.2. Results and Discussion

### 4.2.1. Multivariate Optimisation

The parameter main effects plot (see Figure 4-2) and the parameter interaction plots (see Figure 4-3) give a good visual assessment of the influence the parameters have on the operation of the system and their interactions with each other. From the two plots, optimal conditions can be identified by assessing the position of the centre points and the point where trend lines cross in comparison to the minimum and maximum parameter variables (Minitab, 2008).

***Parameter main effects plots***

A parameter main effects plot is a representation of the mean for each parameter; a line connects the response mean for each factor level. When the line is parallel to the x-axis, there is no main effect present. When the line is not horizontal, then there is a main effect present; the steeper the slope of the line, the greater the magnitude of the main effect (Box *et al.*, 2005; Minitab, 2008).

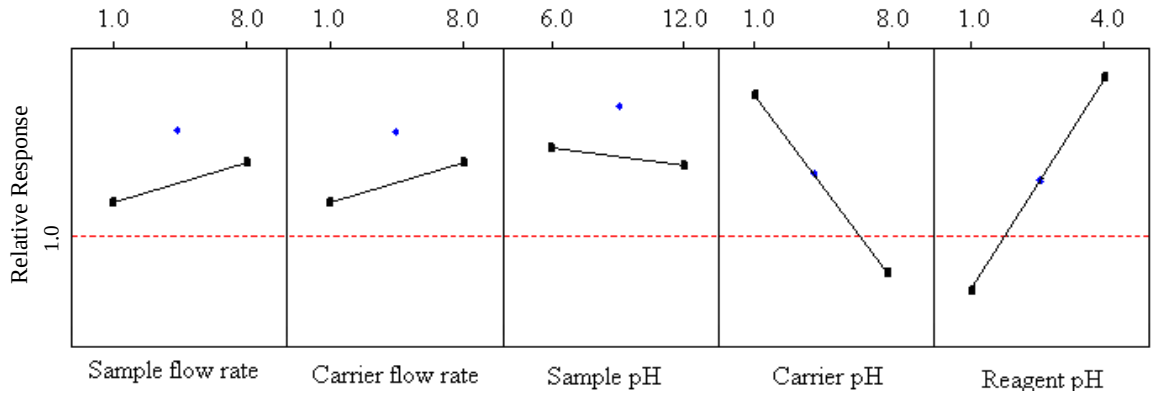



**Figure 4-2: Parameter main effects plot**

From the parameter main effects plot (Figure 4-2), we can identify that both the sample and carrier flow rate increases the chemiluminescence response as the flow rate moves from 1 mL $min^{-1}$ to 8 mL $min^{-1}$. As sample pH moves from 6.0 to 12.0 a decrease in chemiluminescence is observed with a maximum intensity observed at the centre point (9.0). However, because these slopes are small for sample and carrier flow rate, and reagent pH, they are considered to be of little significance within this system.

The steep slope for the carrier and reagent pH parameters indicates that these parameters have a significant effect, with the carrier at pH 1.0 and reagent at pH 4.0 giving the largest response.

### *Parameter interaction plots*

Interactions between the factors can be visualised using an interaction plot. Parallel lines in an interaction plot indicate no interaction, the greater the difference in slope between the lines indicates the higher the degree of interaction (Box *et al.*, 2005; Minitab, 2008). Figure 4-3 illustrates the interaction plot for the selected parameters in the multivariate optimisation.

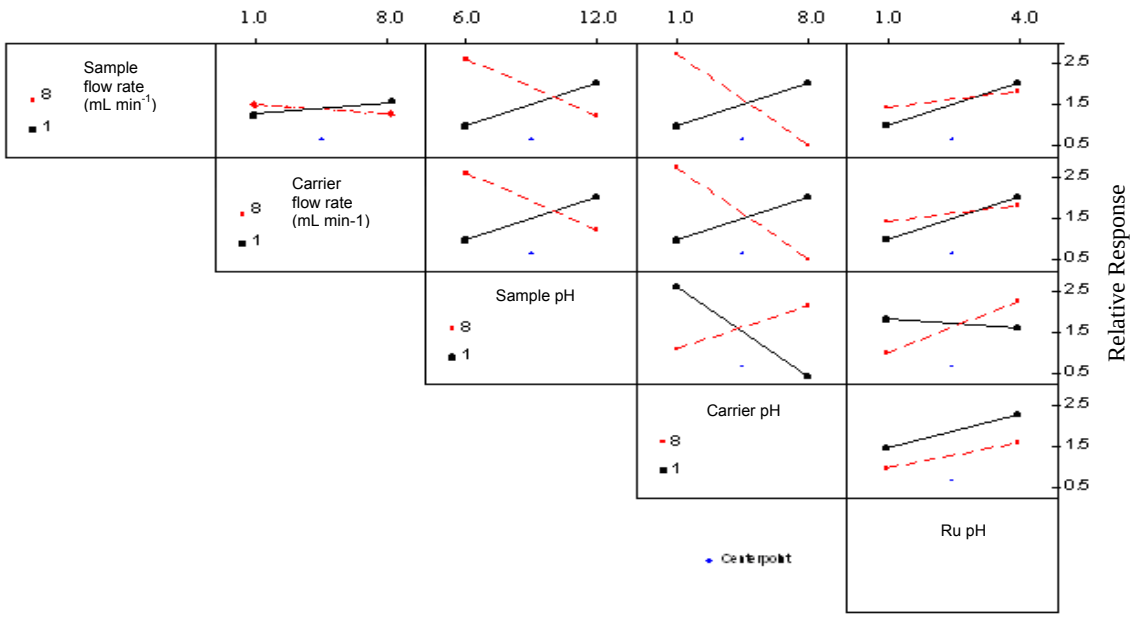



**Figure 4-3: interaction plot for the selected parameters in the multivariate optimisation**

From the interaction plot, it appears that the interaction between reagent pH and carrier pH is negligible (the trend lines are parallel). However, the point of intersection with all the other parameters is at pH 3 for the reagent pH and 4.5-5.0 for the carrier pH. The pH for the reagent is significantly higher than expected (Costin *et al.*, 2004) obtained an optimal reagent pH of 1). However, during the optimisation experiments presented here, it was difficult to keep the reagent oxidised at the higher pH ranges (*i.e.,* pH 3-4).

The sample and carrier flow rate, as was the case in the main effects plot, have a minimal effect on the chemiluminescence response, and the two lines cross at 4.5 mL $min^{-1}$. The point of intersection for the sample pH occurred at pH 10.0.

### *Optimal parameters*

Upon completion of the design experiments, the most suitable optimization parameters (Table 4-4) were selected.

**Table 4-4: Multivariate optimal parameters**

| FICA Parameter | Multivariate Optimal setting |
|---|---|
| Carrier flow rate (mL $min^{-1}$) | 4.5 |
| Sample flow rate (mL $min^{-1}$) | 4.5 |
| Sample pH | 10.0 |
| Carrier pH | 4.5~5.0 |
| Reagent pH | 3 |

Using the data from the multivariate analysis, a univariate optimisation was performed in order to test the robustness of the factors. The interaction plot indicated that sample, carrier and reagent pH all affected the chemiluminescence response, which is supported by the literature. However, the higher than expected reagent pH may have skewed the results for the sample and carrier pH. Detailed below are the outcomes of the univariate design and subsequent optimal values for each parameter chosen.

### 4.2.2. Univariate Optimization

Univariate optimisation was carried out on the flow rate, injection volume, reagent pH, carrier buffer concentration, sample buffer concentration and reagent; the selected parameters were analysed over the working range specified in Table 4-5, the effect of the variable change on the chemiluminescent response was monitored at regular increments. Note, the flow rate of the carrier and sample lines were altered equally (in alignment with the approach undertaken in the multivariate optimisation experiments), the sample buffer concentration and reagent injection volume were added in order to investigate their sensitivity over the range of parameters optimised in the multivariate study.

**Table 4-5: Univariate optimal design parameters and variable range**

| FICA Parameter | Low | High | Number of Increments |
|---|---|---|---|
| Flow rate (mL $min^{-1}$) | 1.5 | 8.6 | 19 |
| Sample pH | 2 | 13 | 9 |
| Carrier buffer concentration (borax) mM | 25.0 | 100.0 | 3 |
| Reagent concentration mM | 1 | 5 | 5 |
| Reagent pH | 1 | 4 | 4 |
| Reagent injection μL | 50.0 | 250.0 | 4 |

### *Effect of flow rate*

The effect of flow rate on the chemiluminescence reaction between atrazine and tris(2'2-bipyridyl)ruthenium(III) was investigated over 1.5 to 8.5 mL $min^{-1}$ (19 increments); all other FICA parameters were maintained at the optimal settings determined in the multivariate optimisation. Figure 4-4 illustrates the effect of flow rate over the entire working range, and Figure 4.5 illustrates the effect of flow rate at 0.2 mL increments between 3.58 to 5.58 mL $min^{-1}$. The highest chemiluminescence signal generated from atrazine and the lowest blank signal (overall highest signal to blank ratio (SBR)) was recorded at 4.6 mL $min^{-1}$ (RSD 0.1%, n=5; 100 µg $L^{-1}$ atrazine). Flow rates ± 1.0 mL $min^{-1}$ resulted in a decreased SBR (>15%) and RSD (>0.5%). *Note: all other parameters were held at the predefined optimal conditions defined in the multivariate optimisation*.

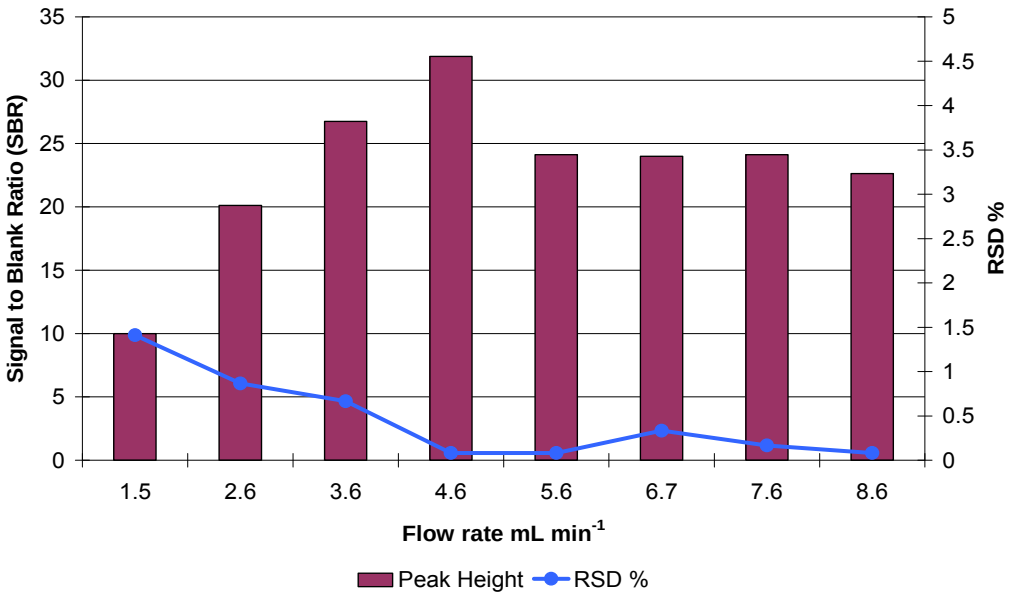



**Figure 4-4: Effect of flow rate on chemiluminescence response (part 1).**

***Note:*** *Investigated effect of flow rate on chemiluminescence response of atrazine (100 µg $L^{-1}$ in MilliQ water) over the working range of 1.5 mL $min^{-1}$ to 8.6 mL $min^{-1}$, at 1.0 to 1.1 mL $min^{-1}$ increments.*

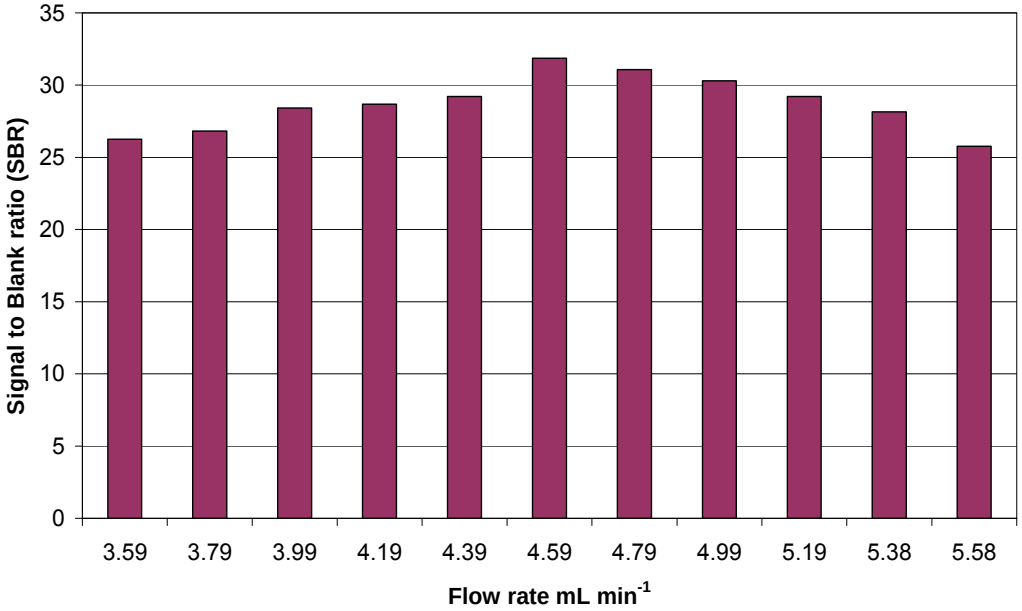



**Figure 4-5: Effect of flow rate on chemiluminescence response (part 2).**

***Note:*** *Investigated effect of flow rate on the chemiluminescence response of atrazine (100 µg $L^{-1}$ in MilliQ water) over the working range of 3.59 mL $min^{-1}$ to 5.58 mL $min^{-1}$, at 0.2 mL $min^{-1}$ increments.*

A variation from the optimal flow rate of ± 0.2 mL $min^{-1}$ resulted in an observed decrease of 2.5% or 8.3% in the analytical response for flow rates of 4.79 and 4.39 mL $min^{-1}$, respectively. It should be noted; the robustness of the flow rate was investigated for atrazine only. While the flow rate was investigated over the same range for the other triazines, it was done at a flow rate increment of 0.5 mL $min^{-1}$ from the optimal flow rate observed for atrazine. It was found that the optimal flow rate for simazine and hexazinone was the same as atrazine, 4.6 mL $min^{-1}$.

### *Effect of sample pH*

Due to the acidic nature of the chemiluminescence reagent, and its reactivity with protonated aliphatic amines, an investigation of analyte pH over the range of 1 – 13 was carried out with freshly prepared buffer solutions (100 µg $L^{-1}$ atrazine in aqueous solution). The buffers used were 0.2 M potassium chloride and 0.2 M hydrochloric acid (HCl) (pH 1-2), 0.1 M potassium hydrogen phthalate and 0.1 M HCl (pH 2-5), 0.1 M potassium dihydrogen phosphate and 0.1 M sodium hydroxide (NaOH) (pH 6-8), 0.25M sodium borate (borax) and 0.1 HCl/ 0.1M NaOH (pH 8-10), 0.1 M sodium carbonate/ 0.1 M sodium phosphate and 0.1 M NaOH (pH 11-12) and potassium chloride and 0.2 M NaOH (pH 13). When the sample pH was below 7 no or minimal chemiluminescence was generated by the target analyte. Above pH 7, the SBR for the reaction significantly increased, reaching maximum chemiluminescence

at pH 9 (borax buffer) before tailing off at pH 13, due to an increase in the blank signal (see Figure 4-6). The sample and carrier flow rate was maintained at 4.6 mL $min^{-1}$; all other parameters were held at the multivariate optimised conditions.

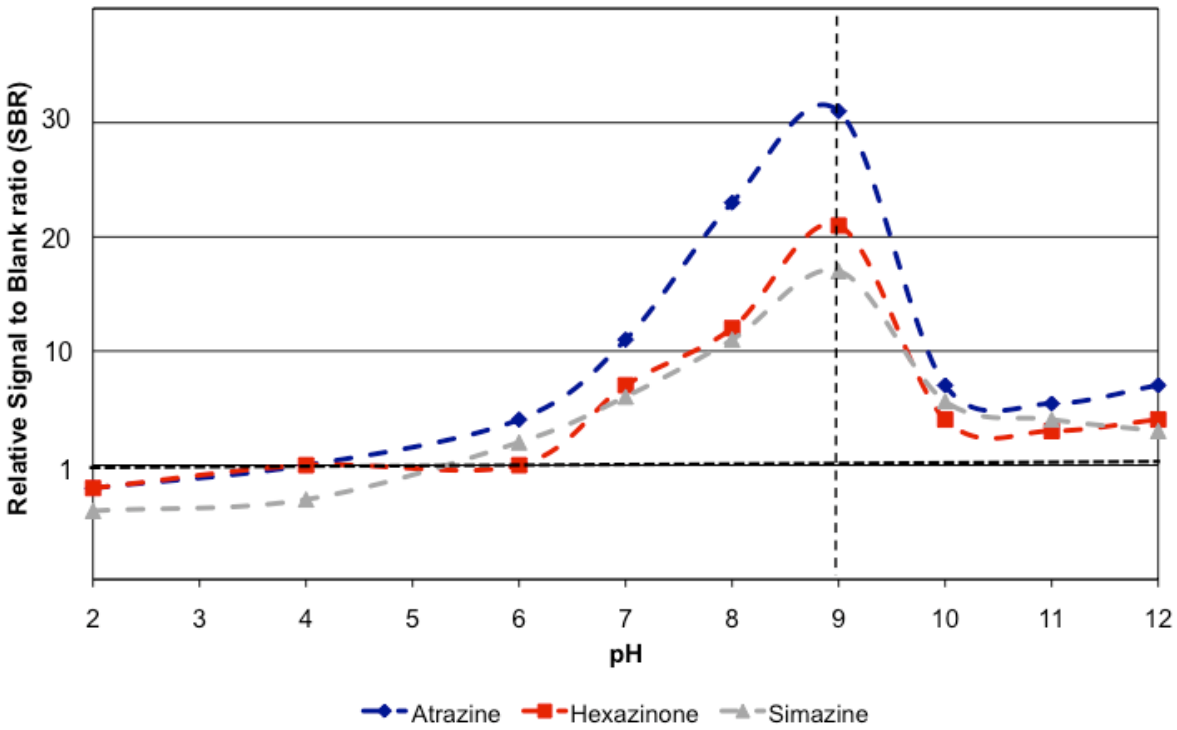



**Figure 4-6: Triazine chemiluminescence variation with change in sample pH.**

The effect of the borax buffer strength was investigated at 25, 50, 100 mM. It was found that the SBR increased 5.6% from a 25 mM to 50 mM borax solution, buffer strength higher than 50 mM had no significant effect on the analyte chemiluminescence generated. Any advantage obtained on the analyte signal was reduced by a greater increase in the blank signal. All subsequent analyses were conducted at pH 9 buffered with 50 mM borax.

### *Effect of tris(2,2'-bipyridyl)ruthenium(III) concentration*

The effect of tris(2,2'-bipyridyl)ruthenium(III) concentration was investigated over the range of 1 to 5 mM; the sample and carrier flow rate was held at 4.6 mL $min^{-1}$ and the sample was maintained at pH 9 . The highest SBR recorded for the reaction with atrazine (50 µg $L^{-1}$ in 50 mM borax buffer pH 9) was when the reagent was 2 mM (SBR 23.6; <5% RSD); there was a 50% reduction in SBR when the reagent was 3 mM (SBR 11.4; <5% RSD). When the concentration was reduced to 1 mM (SBR 22.8; <5% RSD), the SBR was only reduced by 4% from the maximum. Since the overall cost efficiency is greater, as only half the amount of reagent is consumed, all subsequent analyses were performed using 1 mM tris(2,2'-bipyridyl)ruthenium(III).

### *Effect of tris(2,2'-bipyridyl)ruthenium(III) pH*

The pH of tris(2,2'-bipyridyl)ruthenium(III) was investigated over the range of 1 to 4. As was the case in the multivariate optimisation, reagent pH greater than pH 4 was difficult to keep oxidised. As a result reagents at pH 4 and above of tris(2,2'-bipyridyl)ruthenium(III) were not studied in the univariate optimisation. It was found that upon filtration from lead dioxide the reagent was reduced instantly to the inactive tris(2,2'-bipyridyl)ruthenium(II). Despite pH 3 giving the best SBR in the multivariate optimisation analysis, it was found that pH 1 gave more reproducible results (<5% RSD) compared to pH 3 (<23% RSD). The effect of the reagent injection volume was investigated over the range of 50 µL to 250 µL. The injected reagent volume was optimal at 100 µL at a flow rate of 4.6 mL $min^{-1}$; volumes above 100 µL produced broader peaks, with no analytical advantage (*i.e.,* no increase in SBR using the peak area or height). Volumes below 100 µL (*e.g.* 50 µL) had a negative effect on the overall SBR.

## 4.2.3. Optimal FICA operating parameters

From the optimal conditions determined, it is apparent that the multivariate method required a second iteration of design experiments to be created, closing in around the original parameters. This is highlighted by the optimal variables for flow rates and sample pH being defined as equal or near equal to the centre points of the face centred central composite parameters. Box *et al.* (2005) did state that in some cases a second or third iteration of the optimisation design may be required (*i.e.,* slowly closing the range between high, centred and low points within the design) in order to eventually lead to the 'real' optimised parameters. However, the exercise of re-iterations was not required on the basis of utilising information gleaned from the parameter effects plot and interaction plots, in conjunction with the results obtained from the univariate optimisation which further optimised the sample and carrier flow rates, sample and reagent pH. Table 4-6 illustrates the optimized parameters from the multivariate and univariate optimization.

**Table 4-6: Optimized FICA parameters**

| FICA Parameter | Multivariate Optimal setting | Univariate Optimal Setting | Chosen Setting |
|---|---|---|---|
| Carrier flow rate (mL $min^{-1}$) | 4.5 | 4.6 | 4.6 |
| Sample flow rate (mL $min^{-1}$) | 4.5 | 4.6 | 4.6 |
| Carrier pH* | 4.5~5.0 | - | 5.5 (MilliQ water) |
| Sample pH | 10.0 | 9.0 | 9.0 |
| Reagent buffer concentration ($H_2SO_4$) mM* | - | - | 20.0 |
| Sample buffer concentration (borax) mM | - | 50.0 | 50.0 |
| Reagent pH* | 3 | 1 | 1 |
| Reagent concentration | - | 2 (1) | 1 |
| Reagent injection μL | - | 100 | 100.0 |

***NOTE:*** ***optimal reagent concentration selected from the literature; numerous authors have investigated the effect of reagent pH and it has been determined that 20 mM $H_2SO_4$ is optimal (e.g. Costins et al.,2004; and Gorman et al., 2006 etc.).*

### *Flow cell volume and chemiluminescence reaction time (validation calculation)*

As previously stated, tris(2,2'-bipyridyl)ruthenium(III) is known to react with aliphatic amines and light is produced (*i.e.,* chemiluminescence); the rate of this reaction is determined by the analyte and the reaction sites involved. Beale (2004) used pulse flow analysis (*e.g.* simultaneously injecting tris(2,2'-bipyridyl)ruthenium(III) and 50 μM atrazine (pH 9) into a mixing chamber pushed against a PMT) to determine the reaction half life of tris(2,2'-bipyridyl)ruthenium(III) with atrazine. The reaction half life was determined to be 0.05 seconds, *Note: in comparison, similar analytes (i.e., L-proline, a secondary aliphatic amine) were observed to have a half life of 0.4 seconds*.

Using the reaction half life determined by Beale (2004), the volume and position of the reacting sample plug can be determined and an assessment of the flow cell can be made.

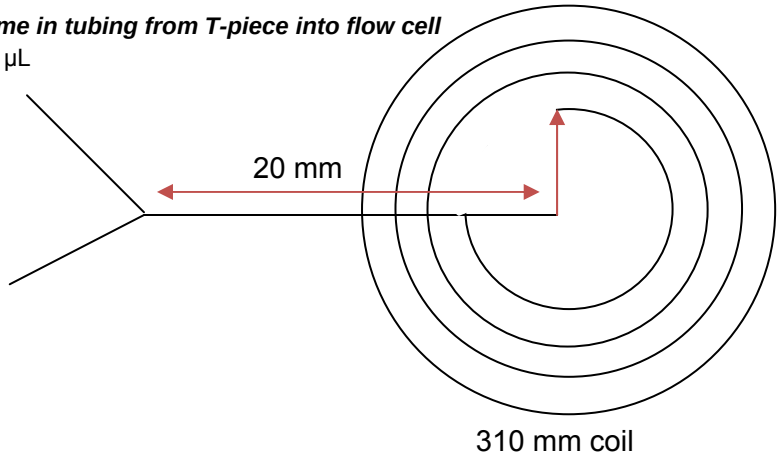
As illustrated in Figure 4-7, for a flow cell comprising 310 mm (0.5 i.d.) tubing with a 20 mm (0.5 i.d.) piece of tubing from the T-piece into the flow cell operated at a flow rate of 4.6 mL $min^{-1}$ (8.2 mL $min^{-1}$ combined flow rate), the volume captured is *ca*. 3 μL (this is the volume of the reaction in the flow cell prior to the reaction half life). As the reaction flows through the flow cell, it comprises *ca.* 58 μL of the sample after the reaction half life has been reached.

**Flow Cell Volume**

***Injection volume***
= 100 µL

***Flow cell volume***
= 60.9 µL

***Volume in tubing from T-piece into flow cell***
= 3.9 µL




**Reaction Rate:**

***Reaction half life***
= 0.05 seconds

***Volume travelled before reaction half life ($t_{1/2}$)***
= 6.8 µL

***Volume travelled ($t_{1/2}$) minus volume in tubing from T-piece to flow cell (volume in cell)***
= 3.0 µL

***Reaction captured in flow cell***
= 3.0 µL prior to reaction half life
= 57.9. µL after reaction half

**Figure 4-7: Flow cell volume and chemiluminescent reaction rate**

## 4.3. Determination of Atrazine in MilliQ water

Atrazine solutions over the concentration range 1.15 µg $L^{-1}$ – 10.5 mg $L^{-1}$ were prepared in 50 mM borax at pH 9 and analysed using the parameters listed in Table 4-6. Table 4-7 summarises the analytical figures of merit achieved for the determination of atrazine by flow injection chemiluminescence (n=4) (see Appendix C for the atrazine calibration graph and FICA trace of atrazine standards). The log-log calibration function was Y=mX+c; where Y is the response measured (mV) and X is the concentration in concentration units (µg $L^{-1}$), m is the slope and c is the intercept.

MilliQ water spiked with increasing amounts of atrazine (30.0, 50.0 and 100.0 µg $L^{-1}$) was analysed using the same method as above and the results were confirmed using direct HPLC (Perkins *et al.*, 1999). Table 4-8 reports observed concentrations and recovery for both analytical methods. The recoveries for both methods were excellent, with the FICA method giving slightly higher calculated recoveries than the HPLC method.

**Table 4-7: Determination of atrazine in MilliQ water (figures of merit)**

| Statistical Figures of Merit | |
|---|---|
| Intercept | -0.0944 |
| Slope | 0.1515 |
| Linear dynamic range (µg $L^{-1}$) | 1.15 – 2150 |
| Limit of Detection (LOD)[a] (µg $L^{-1}$) | 1.3 |
| Limit of Quantification (LOQ)[b] (µg $L^{-1}$) | 3.1 |
| Practical Method detection Limit (MDLP)[c] (µg $L^{-1}$) | 2.0 |
| Standard Deviation of the Slope | 0.003 |
| Standard Deviation of the Intercept | 0.01 |
| Standard Deviation of the Regression | 0.03 |
| Correlation Coefficient | 0.9795 |

***NOTE:*** *[a]Limit of Detection calculated using 3 x SD of the blank (n=36 (2005; 2008b). [b]Limit of Quantification calculated using 10 x SD of the blank (n=36) (2008b). [c]Practical Method Detection Limit calculated using 1.15 µg $L^{-1}$ atrazine (n=8), the standard deviation was calculated and multiplied by the one-sided t distribution (Long & Winefordner, 1983).*

**Table 4-8: Recovery for MilliQ water samples spiked with atrazine using the flow injection chemiluminescence and direct injection HPLC methods**

| Analytical method | [Atr] spike (µg $L^{-1}$) | [Atr] observed ± 2sd. (µg $L^{-1}$) | Recovery (%) |
|---|---|---|---|
| Flow injection | 30.0 | 32.1 ± 0.1 | 107 |
| | 50.0 | 51.8 ± 0.3 | 104 |
| | 100.0 | 102.6 ± 0.3 | 103 |
| HPLC direct injection | 30.0 | 29 ± 4 | 97 |
| | 50.0 | 48 ± 2 | 96 |
| | 100.0 | 99 ± 3 | 99 |

***Note:*** *the same samples were analysed by FIA and HPLC; n=4.*

A limit of detection (LOD) of 1.3 ± 0.1 µg $L^{-1}$ in MilliQ water without pre-concentration for atrazine was was achieved; in comparison to the methods described in Table 2-5, this is considered high. For example, Tudorache *et al.* (2008) obtained a LOD of 0.003 µg $L^{-1}$ in water by employing an immunoassay chemiluminescence technique with pre-concentration. Schobel *et al.* (2000) obtained a LOD of 0.1 µg $L^{-1}$ using immunoassay fluorescence, Jiang *et al.* (1995) used an electrode to obtained a LOD of 0.1 µg $L^{-1}$, Bjarnason *et al.* (1997) applied a UV detector with a LOD of 0.5 µg $L^{-1}$ and Hassan *et al.* (1998) used a potentiometric detector for a LOD 0.3 µg $L^{-1}$. However, it should be noted that the method described here did not have a pre-concentration step.

## 4.4. Conclusions

A multivariate and univariate optimisation method was applied to the FICA instrument, and the developed optimised experimental conditions were determined to be: sample and carrier flow rates of 4.6 mL $min^{-1}$, sample at pH 9 buffered with 50 mM borax, and a reagent concentration of 1 mM tris(2,2'-bipyridyl)ruthenium(III) in 20 mM $H_2SO_4$ (pH 1).

Atrazine was detected by flow injection chemiluminescence using tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence with a limit of detection of 1.3 ± 0.1 µg $L^{-1}$ in MilliQ water without pre-concentration. Validation of the method was performed by direct injection HPLC, with no significant difference observed between the methods. ($R^2$ = 0.9906, t-test (6) = 0.39 (p two tailed = 0.71) and -0.74 (p two tailed = 0.48) for 0.5 and 10 µg $L^{-1}$ atrazine respectively).

In the upcoming chapters, the system described in this chapter is applied to the determination of atrazine in natural waters, as well as an investigation into the influence of possible interfering compounds and other pesticides (see Chapter Seven). The following chapter describes the development and optimisation of a direct injection HPLC method used to compare subsequent FICA analysis.

# CHAPTER FIVE: OPTIMISATION OF THE HPLC VALIDATION METHOD

As discussed in Chapter Two, many researchers have investigated a variety of methods for the determination of triazines and triazinones in numerous aqueous samples (see review by McLaughlin *et al.*, 2008). Such methods include: HPLC with ultraviolet (UV) or electrospray ionisation mass spectrometry (ESI-MS) detection, ultra performance liquid chromatography (UPLC) MS, gas chromatography (GC) with flame ionisation detection (FID), nitrogen phosphorus detection (NPD) or MS, thin layer chromatography, capillary electrophoresis (CE), hyphenated chromatography (*e.g.* LC/GC-MS) and immunoassays techniques (*e.g.* ELISA methods).

The purpose of the work presented in this Chapter was to develop a validation method that can be used to compare the FICA methods described herein (both in terms of analytical performance, analysis time, and ease of operation). While current standard methods recommended for the determination of pesticide residues are satisfactory in regard to detection limits and analytical performance, they are time consuming. Numerous researchers have investigated alternate techniques that are less time consuming; one approach involves the development of various liquid chromatography techniques, in particular HPLC, UPLC and LC as shown in Table 5-1.

**Table 5-1: Summary of recent developments in rapid pesticide analysis by HPLC.**

| Method | Analyte | Extraction type | Retention time ($T_R$) (minutes) | Limit of Detection (LOD) (µg $L^{-1}$) | Reference |
|---|---|---|---|---|---|
| HPLC UV | Atrazine | SPE | 34 | - | JunkerBuchheit & Witzenbacher, 1996 |
| | Simazine | | 23 | | |
| HPLC–UV | Atrazine | Liquid | 10 | 0.1 | Zhou *et al.*, 2008; Zhou *et al.*, 2009 |
| | Simazine | | 12.6 | 0.04 | |
| LC-ES-MS | Atrazine | SPE | 2.37 | 0.0006 | Mezcua *et al.*, 2006 |
| | Simazine | | 1.91 | 0.00008 | |
| UPLC-ESI-MS/MS | Atrazine | SPE | 2.3 | 0.006 | Gervais *et al.*, 2008 |
| | Simazine | | 1.6 | 0.01 | |
| HPLC-UV | Hexazinone | Direct injection | 7.9 | 0.3 | Perkins *et al.*, 1999 |
| LC-EIS-MS | Atrazine | Direct injection | 4.39 | 0.3 | Diaz *et al.*, 2008 |
| | Simazine | | 4.36 | | |

Although LC-MS methods offer significant reductions in detection limits, and considerable effort has been expended to reduce retention times (retention times commonly achieved between *ca.* 2 to 5 minutes for atrazine and simazine (Diaz *et al.*, 2008; Mezcua *et al.*, 2006), they are considered to be highly sophisticated and expensive pieces of equipment requiring specialized personnel to operate and interpret MS data. In contrast, conventional HPLC-UV methods are typically more robust, cheaper and easier to operate, but fall short in terms of required detection limits and the time required for analysis. However, recent advancements in monolithic column technology have lead to an improvement in peak resolution, and combined with HPLC, provide an option for fast screening of samples prior to conventional methods, making it a suitable method for comparison with FICA.

This chapter describes the development of a modified large volume direct injection HPLC method utilizing a monolithic column. The HPLC method described provides a platform to evaluate the application of monolithic columns for pesticides analysis and assess their suitability for integration into FICA methods (discussed in more detail in Chapter Nine). The integration of a monolithic column in FICA would enable multiple analytes to be analysed, increasing the analytical performance of conventional FIA systems while being significantly cheaper than HPLC. In general, resolution between solute bands depends on the square root of column efficiency (*i.e.*, the number of theoretical plates (NTP)), which in turn is proportional to the reciprocal of particle diameter ($1/d_p$) of the column packing material.

Concurrently, the pressure drop across the column is inversely proportional to the square of $d_p$. Attempts to obtain greater NTP by decreasing particle size, results in significant increases in instrument operational pressure, which often exceeds the instrument specifications. The structure of monolithic columns overcomes this problem (Diaz *et al.*, 2008).

Monolithic columns are prepared by in-situ polymerization of monomers in a column, providing greater flexibility than densely packed columns, and a wider range of monomers can be used with integrated structures that can increase the overall porosity. The higher porosity leads to an increase in permeability which consequently results in a decrease in the required operational pressure. Coupled with the presence of small-sized mono structure skeleton, higher efficiencies can be expected. Up to now, monolithic columns have been used mainly for the determination of amino acids and drug residues (Ochsenbein *et al.*, 2008), although there have been some instances of modified chromatography flow injection and sequential injection techniques for pesticide analysis (*e.g.* Chocholous *et al.*, 2008).

First, the development of the proposed method is described and compared to a conventional packed column, along with its application to natural waters. This is followed with an investigation into possible interferences from triazine metabolites.

## 5.1. Experimental

### 5.1.1. Sample and Solution Preparation

All samples and solutions were prepared as detailed in Chapter Three.

### 5.1.2. Direct Injection HPLC

The HPLC system previously described in Chapter Four was modified by substituting the packed separation column with a monolithic column. A Waters HPLC system (M-6000A, Waters Associates Inc., USA.) operated isocratically was used. Aqueous samples were injected (500 µL) via a Waters HPLC injection valve fitted with a 500 µL loop using a 2 mL glass barrel syringe. The injected sample passed through a monolithic column RP-18e, 50-4.6 mm (Chromolith, Merck). The HPLC system was connected to a UV–vis detector (SPD-10AV, Shimadzu, Japan) set at 220 nm coupled to a chart recorder (Model 3395, Hewlett Packard, USA) and a personal computer operating ChemStation (Agilent, USA).

## 5.2. Results and Discussion

### 5.2.1. Mobile Phase Optimisation

A mobile phase method development triangle was created according to Harris (1997) (Merck, 2009). Method development triangles are a systematic process applied in HPLC to develop a mobile phase suitable for the separation of analytes using a combination of solvents (MeOH, methanol; ACN, acetonitrile; and MilliQ water). Organic solvents varied in composition along the development triangle between 10-90% at regular intervals (see Appendix D for the development triangle details and resulting effect on the separation efficiency). The mobile phase chosen for HPLC analysis was 30:70% ACN:$H_2O$. While mobile phases comprising various combinations of MeOH, ACN and water were all effective for analysing the individual triazine compounds, a problem of co-eluting hexazinone and simazine peaks occurred when all three compounds were analysed together; this was overcome utilising 30:70% ACN:$H_2O$.

### 5.2.2. Effect of Mobile Phase Flow Rate

The effect of the mobile phase flow rate on the direct injection HPLC analysis of atrazine, hexazinone and simazine was investigated over 0.25 – 3.00 mL $min^{-1}$ in 0.25 mL $min^{-1}$ increments. However, when using the monolithic column the mobile phase flow rate did not significantly influence the instrument operating pressure or the quality of the chromatography (*i.e.*, peak width and resolution). A flow rate of 2.0 mL $min^{-1}$ was selected for all subsequent experiments, since it was the minimum flow rate which achieved baseline separation between all analytes selected and it was at a rate which could be compared with a C8, 5 µm, 250 mm x 4.6 mm column (model 831815 Spherisorb, Phase Separations, USA); the packed column was used with a mobile phase flow rate of 1.5 mL $min^{-1}$.

### 5.2.3. Effect of Injection Volume

The effect of the sample injection volume on the direct injection HPLC analysis of atrazine, hexazinone and simazine was investigated over 100 – 1000 µL. It was found that the analyte peak area steadily increased as the volume increased from 100 µL to 1000 µL; however, the optimal peak shape was achieved using 500 µL. Injection volumes larger than 500 µL distorted the symmetry of the peak as well as causing peaks to become broader. All subsequent analysis was performed using a filled 500 µL sample injection loop.

### 5.2.4. Effect of Detector Wavelength

A number of researchers have investigated atrazine and simazine using detection at wavelengths between 220 and 223 nm, and hexazinone at 244 nm (Mouvet *et al.*, 1997; Perkins *et al.*, 1999). As illustrated in Figure 5-1, the lambda maximum of atrazine and simazine was found to be 220 nm while the lambda maximum of hexazinone was 244 nm; for reference, the spectrum of a water sample comprising 1 mg $L^{-1}$ of dissolved organic carbon is also presented (samples consisting DOM concentrations higher than 1 mg $L^{-1}$ saturated the detector of the spectrophotometer). While natural waters can be found with DOM concentrations significantly higher than 1 mg $L^{-1}$ water sample presented (*e.g.* as high as 14 mg $L^{-1}$), Figure 5-1 illustrates both atrazine and simazine are susceptible to interference by the tail of the DOM spectra. It is also noteworthy that while 220 and 244 nm is suitable for direct injection analysis for atrazine and simazine, and hexazinone respectively; any interference caused by DOM can be eliminated through efficient separation on the HPLC column.

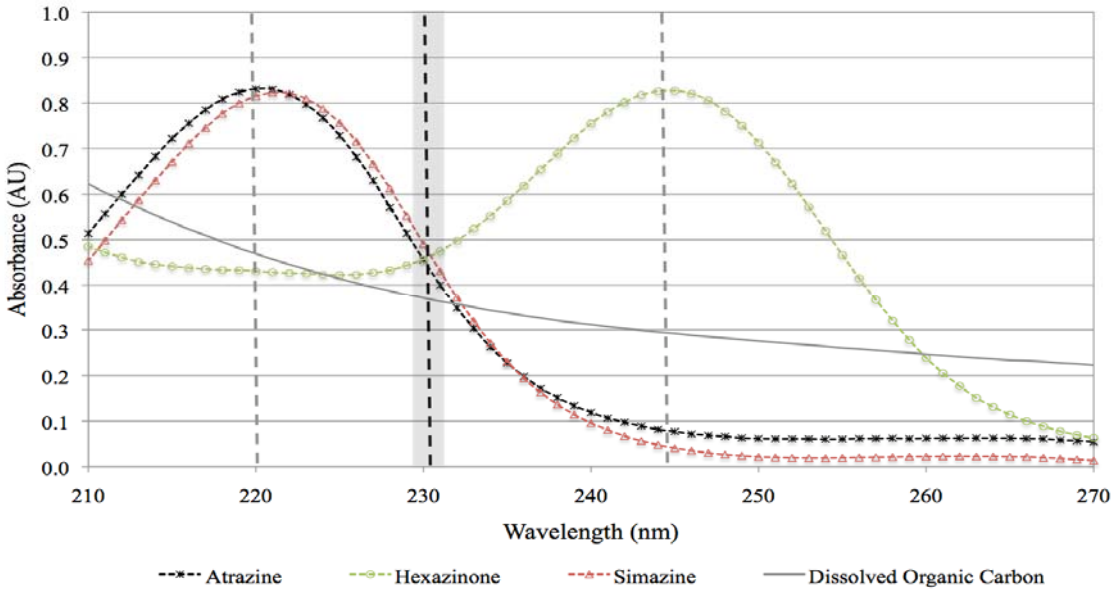



**Figure 5-1: UV spectra of atrazine, hexazinone, and simazine**

***Note:*** *UV spectra of atrazine, hexazinone, and simazine (500 µg $L^{-1}$) in MilliQ water and a 1 mg $L^{-1}$ DOM natural water sample (DCM sample diluted 1:10 with milliQ water).*

## 5.3. Determination of Atrazine, Simazine and Hexazinone in MilliQ Water

Pesticides at standard concentrations over the range 5-50 µg $L^{-1}$ were prepared in MilliQ water and analysed using the best operating conditions determined: sample injection

volume of 500 µL, mobile phase of 30:70 ACN:$H_2O$ at 2 mL $min^{-1}$ with detector wavelengths of 220 and 244 nm. The analytical and statistical parameters obtained for the determination of the three pesticides by direct injection HPLC are summarised in Table 5-2. The calibration function was Y=mX+c; where Y is the response measured (area) and X is the concentration in concentration units (µg $L^{-1}$), m is the slope and c is the intercept. Figure 5-2 illustrates the difference in chromatographic separation over the two different wavelengths using the monolithic column. Figure 5-3 illustrates the chromatographic differences between the monolithic column and the packed column.

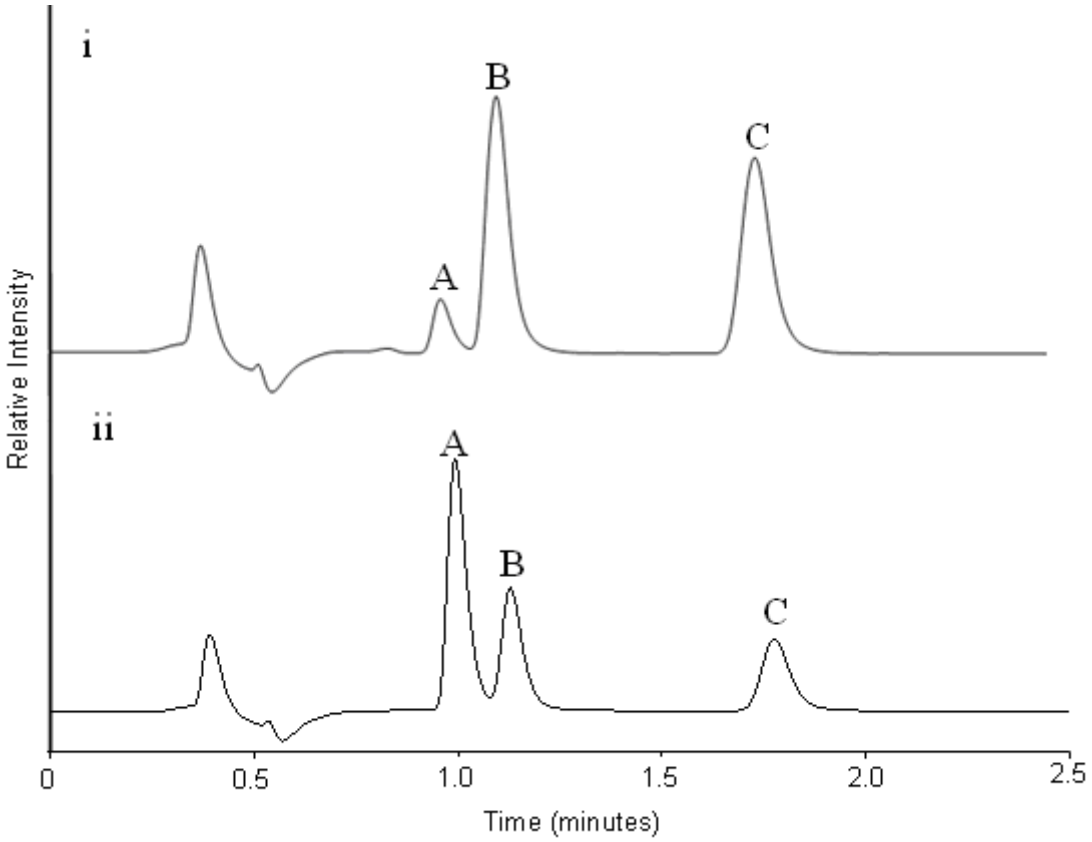



**Figure 5-2: HPLC monolithic chromatogram of atrazine, simazine and hexazinone.**

***NOTE:*** *Chromatogram of 50 µg $L^{-1}$ standard solution of atrazine, simazine and hexazinone at 220 and 244 nm; peaks identified as (A) hexazinone; (B) simazine; (C) atrazine. Sample solution consisted of 50 µg $L^{-1}$ pesticide in MilliQ water, 500 µL injection. Mobile phase 30:70 (ACN:$H_2O$) with a UV-vis detector (i= λ 220 nm; ii= λ 244 nm), flow rate 2.0 mL $min^{-1}$.*

**Table 5-2: Summary of analytical figures of merit for the determination of atrazine, simazine and hexazinone in MilliQ water by direct injection HPLC for packed and monolithic columns.**

| Statistical figures of merit | UV-vis Detector Wavelength | | | | | |
|---|---|---|---|---|---|---|
| | 220 nm | | | | 244 nm | |
| | Atrazine | | Simazine | | Hexazinone | |
| | Mono.[d] | Pack.[e] | Mono.[d] | Pack.[e] | Mono.[d] | Pack.[e] |
| Slope | 6979 | 6907 | 7793 | 7459 | 8724 | 9413 |
| Intercept | -3370 | -536 | -13483 | 3413 | 645 | 502 |
| Linear dynamic range (µg $L^{-1}$) | 5-50 | 5-50 | 5-50 | 5-50 | 1-50 | 0.5-50 |
| LOD[a] (µg $L^{-1}$) | 0.5 | 0.4 | 0.6 | 1.0 | 1.0 | 0.3 |
| LOQ[b] (µg $L^{-1}$) | 1.6 | 1.2 | 0.9 | 4.7 | 2.2 | 1.2 |
| MDL[c] (µg $L^{-1}$) | 1.1 | 1.4 | 1.2 | 3.0 | 0.9 | 1.1 |
| SD of the intercept | 4916 | 6180 | 14221 | 3891 | 524 | 1289 |
| SD of the slope | 143 | 200 | 280 | 126 | 246 | 245 |
| SD of the regression | 6347 | 9598 | 19389 | 6043 | 719 | 2412 |
| Correlation coefficient | 0.9978 | 0.9976 | 0.9921 | 0.9991 | 0.9976 | 0.9973 |

***Note:*** *[a]Limit of detection (LOD) calculated using 3 x SD of the Blank (n=4). [b]Limit of quantification (LOQ) calculated using 10 x SD of the Blank (n=4). [c]Practical method detection limit (MDL) calculated using lowest standard (n=8), the standard deviation (SD) was calculated and multiplied by the one sided t distribution (95%). [d]HPLC system with monolithic separation column (Mono), retention time(s) for 220/244 nm: atrazine 1.77 mins; simazine 1.12 mins; hexazinone 0.98 mins. [e]HPLC system (Packed) devised by Perkins et al. operated isocratically with a $H_2O$:ACN:MeOH (60:25:15) mobile phase at a flow rate of 1.5 mL $min^{-1}$; and retention time (s) for 220/244 nm: atrazine 6.12 mins; simazine 4.52 mins; hexazinone 3.98 mins.*

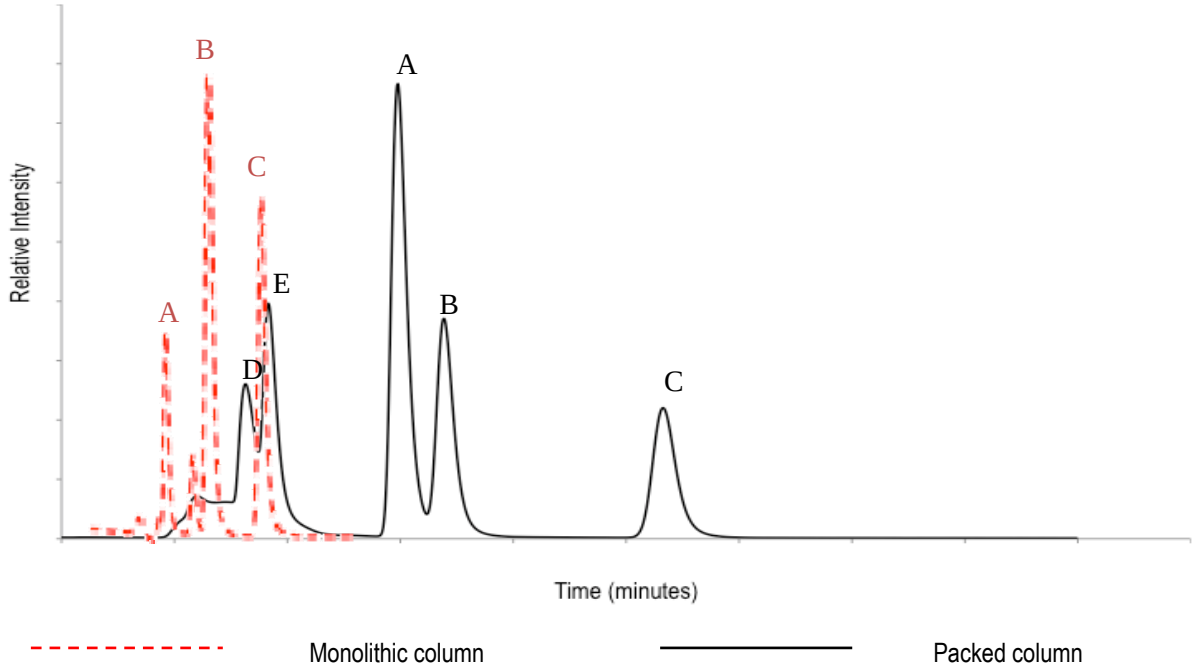



**Figure 5-3: HPLC monolithic and packed column chromatogram of atrazine, simazine and hexazinone**

***NOTE:*** *Chromatogram of 50 µg $L^{-1}$ standard solution of atrazine, simazine and hexazinone. Monolithic column chromatogram (red) was recorded at 220 while the packed column chromatogram was recorded at 244 nm; peaks identified as (A) hexazinone; (B) simazine; (C) atrazine; (D) monocrotophos and (E) dicrotophos. Sample solution consisted of 50 µg $L^{-1}$ pesticide in MilliQ water, 500 µL injection. Mobile phase 30:70 (ACN:$H_2O$) with a UV-vis detector (λ 220 nm or λ 244 nm).*

## 5.4. Determination of Monocrotophos and Dicrotophos in MilliQ Water

Standard concentrations of monocrotophos and dicrotophos over the range 5-50 µg $L^{-1}$ were prepared in MilliQ water and analysed using the best operating conditions determined. The analytical and statistical parameters obtained for the determination of the two pesticides by direct injection HPLC are summarised in Table 5-3.

The method was validated using a series of analytical standards. The analytical performance for the packed and monolithic columns are comparable (*i.e.* within ± 0.7 µg $L^{-1}$ at 220 and 244 nm). The main advantage of the monolithic column over the packed column is the reduction in retention time for each analyte, resulting in a significantly shorter analysis time. The structure of the monolithic column enables higher flow rates to be achieved while operating at a lower pressure. For example, at a flow rate of 1.7 mL $min^{-1}$ using the packed column created an operating pressure of 3500 psi and an analysis time of 7 minutes per sample compared to 3.0 mL $min^{-1}$ for the monolithic column at an operating pressure of *ca.* 500 psi and analysis time under 2 minutes per sample.

**Table 5-3: Summary of analytical figures of merit for the determination of monocrotophos and dicrotophos in MilliQ water by direct injection HPLC for packed and monolithic columns.**

| Statistical figures of merit | UV-vis Detector Wavelength 220 nm | | | |
|---|---|---|---|---|
| | Monocrotophos | | Dicrotophos | |
| | Mono.[d] | Pack.[e] | Mono.[d] | Pack.[e] |
| Slope | 8.8 | 2.5 | 645 | 0.1241 |
| Intercept | 12.5 | 10.9 | 8724 | 0.0314 |
| Linear dynamic range (µg $L^{-1}$) | 4.5-100 | 4.5-100 | 0.6-100 | 5-100 |
| LOD[a] (µg $L^{-1}$) | 2.0 | 1.0 | 0.2 | 0.3 |
| LOQ[b] (µg $L^{-1}$) | 8.2 | 3.9 | 0.8 | 1.9 |
| MDL[c] (µg $L^{-1}$) | 1.6 | 0.6 | 1.2 | 2.2 |
| SD of the intercept | 0.5 | 4.4 | 246 | 0.0004 |
| SD of the slope | 7.4 | 0.2 | 524 | 0.0007 |
| SD of the regression | 11.1 | 5.0 | 719 | 0.005 |
| Correlation coefficient | 0.9949 | 0.9989 | 0.9976 | 0.9992 |

***Note:*** *[a]Limit of detection (LOD) calculated using 3 x SD of the Blank (n=4). [b]Limit of quantification (LOQ) calculated using 10 x SD of the Blank (n=4). [c]Practical method detection limit (MDL) calculated using lowest standard (n=8), the standard deviation (SD) was calculated and multiplied by the one sided t distribution (95%). [d]HPLC system with monolithic separation column (Mono), retention time(s) for 220 nm: monocrotophos 0.55 mins; dicrotophos 0.39 mins. [e]HPLC system (Packed) devised by Perkins et al. operated isocratically with a $H_2O$:ACN:MeOH (60:25:15) mobile phase at a flow rate of 1.5 mL $min^{-1}$; and retention time (s) for 220 nm: monocrotophos 3.9 mins; dicrotophos 2.90 mins.*

### 5.4.1. Application to Natural Samples

To test the effect of natural organic matter (referred to as dissolved organic carbon; DOM) on the analytical performance of the described direct injection HPLC method, six water samples collected throughout Victoria, Australia in 2006, with various DOM concentrations were analysed (as described in Table 5-4). Samples were stored at 4°C; all samples were brought up to room temperature (*c.a.* 22°C) prior to analysis. The natural water samples were free from pesticide residue.

**Table 5-4: summary of characteristics of natural water samples analysed by direct injection HPLC**

| Sample ID tag | Water source | DOM† (mg $L^{-1}$) | Location in Victoria | Primary land activity |
|---|---|---|---|---|
| 1 | Ground water | 3.1 | South East | Cattle farm |
| 2 | Creek (seasonal) | 4.5 | South East | Livestock |
| 3 | river (metropolitan) | 6.5 | Central | Metropolitan |
| 4 | Drinking water catchment | 10.7 | South West | Agriculture |
| 5 | Drinking water catchment* | 11.1 | East | Natural reserve |
| 6 | Drinking water catchment | 11.7 | South West | Agriculture |

***Note:*** **Decommissioned drinking water catchment, primary land activity is closed to public access natural reserve. †Dissolved organic carbon (DOM) measured using a total organic carbon analyser.*

The presence of organic matter was apparent in direct injection HPLC chromatograms. Samples directly injected into the HPLC without pre treatment showed a distinctive DOM peak within the first 0.7 min compared with the standards (see Figure 5-4). The presence of DOM was confirmed by 3DEEM fluorescence spectroscopy, where distinct humic and fulvic acid fluorophores were observed at 237-260/400-500 and 300-370/400-500 (excitation/emission wavelength), respectively, for all samples (Perkins *et al.*, 1999; Trajkovska *et al.*, 2001; Baranowska *et al.*, 2006).

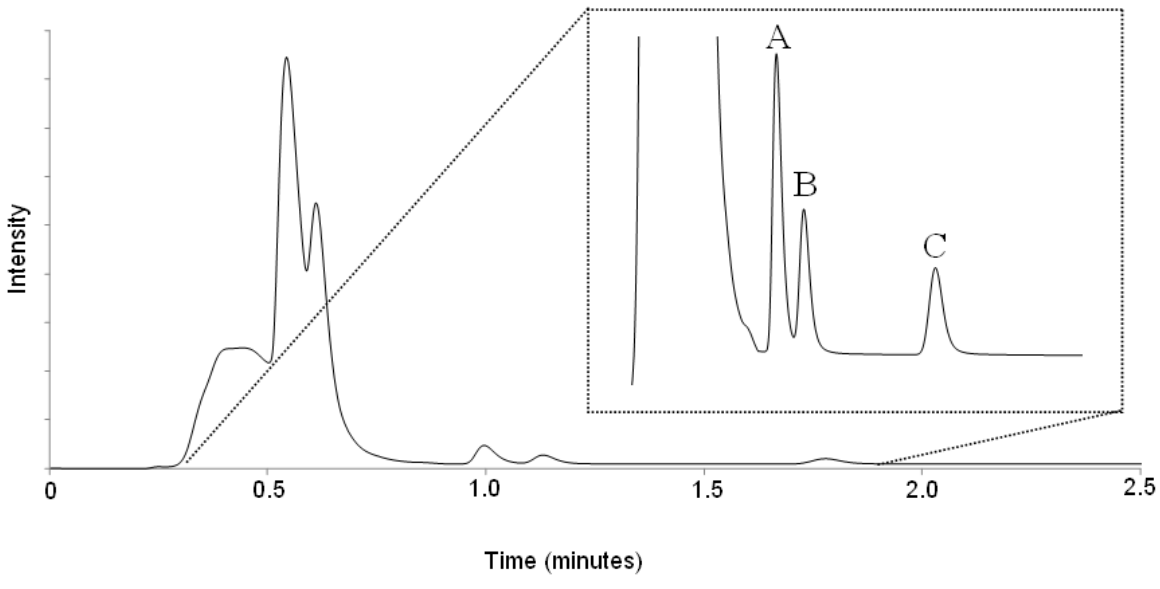



**Figure 5-4: Effect of natural organic matter on chromatography performance.**

***NOTE:*** *Natural organic matter defined as dissolved organic matter (DOM); peaks identified as (A) hexazinone; (B) simazine; (C) atrazine. Sample number three; DOM = 6.5 mg $L^{-1}$) spiked with 10 µg $L^{-1}$ pesticide, 500 µL injection. Mobile phase 30:70 (ACN:H2O) with a UV-vis detector (λ 244 nm), flow rate 3.0 mL $min^{-1}$.*

The natural waters spiked with increasing amounts of atrazine, simazine, hexazinone, monocrotophos and dicrotophos were analysed using the direct injection method at a

wavelength (λ) of 220 nm and 244 nm. The position of the DOM peak did not interfere with those of atrazine, simazine and hexazinone in any of the samples analysed ($p<0.05$ at 95% confidence interval). However, the DOM peak co-eluted with monocrotophos and dicrotophos when tested (see Figure 5-5). As such, monocrotophos and dicrotophos were not were not analysed by direct injection HPLC in the succeeding chapters. Table 5-5 reports the recovery and relative standard deviation; statistical analysis indicates strong correlation between expected and observed concentrations for atrazine, simazine and hexazinone.

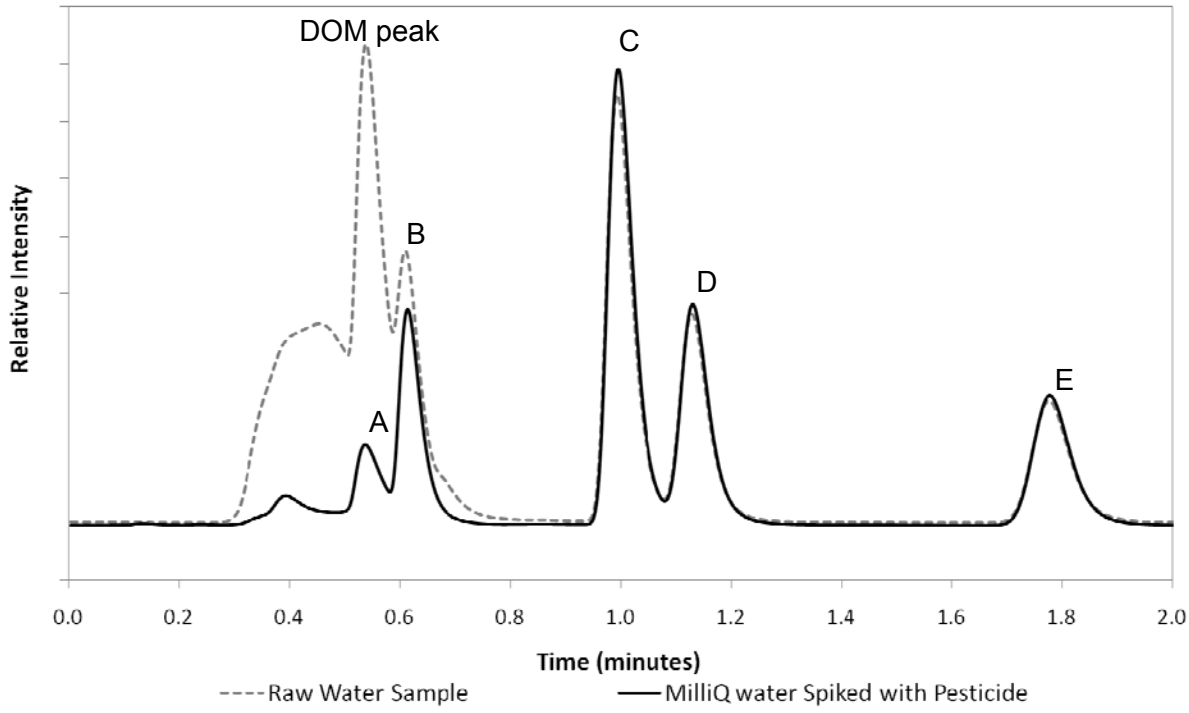



**Figure 5-5: Direct injection HPLC analysis of atrazine, hexazinone and simazine Monocrotophos and dicrotophos analysis by HPLC**

***NOTE:*** *peaks identified as (A) dicrotophos; (B) monocrotophos; (C) hexazinone; (D) simazine; (E) atrazine. Sample solution consisted of 50 µg $L^{-1}$ pesticide in milliQ water, 500 µL injection. Mobile phase 30:70 (ACN:$H_2O$) with a UV-vis detector ( λ 220 nm), flow rate 3.0 mL $min^{-1}$. DOM concentration ca. 1 mg $L^{-1}$.*

**Table 5-5: Direct injection HPLC analysis of atrazine, hexazinone and simazine.**

| | Recovery % (%$R_{SD}$) | | |
|---|---|---|---|
| **Concentration (µg $L^{-1}$)** | **Atrazine (220 nm)** | **Hexazinone (244 nm)** | **Simazine (220 nm)** |
| 10.0 | 96 (4.4) | 95 (6.1) | 94 (6.3) |
| 50.0 | 99 (4.7) | 101 (5.5) | 98 (3.6) |
| **Statistical figures of merit** | | | |
| Pearson Correlation coefficient | 0.9925 | 0.9963 | 0.9867 |
| p-value | 0.83 | 0.87 | 0.97 |
| Correlation coefficient | 0.9961 | 0.9943 | 0.9893 |

### 5.4.2. Interferences

The interference from '*like*' compounds, in terms of peak resolution and retention time, was investigated utilising known atrazine metabolites, as well as other known triazine pesticides. Figure 5-6 illustrates the co-elution of peaks between simazine and the metabolite atrazine-2-hydroxy (Figure 5-6 (i)); this was observed for both the monolithic and packed columns. Other triazines analysed were not observed to co-elute under the described conditions.

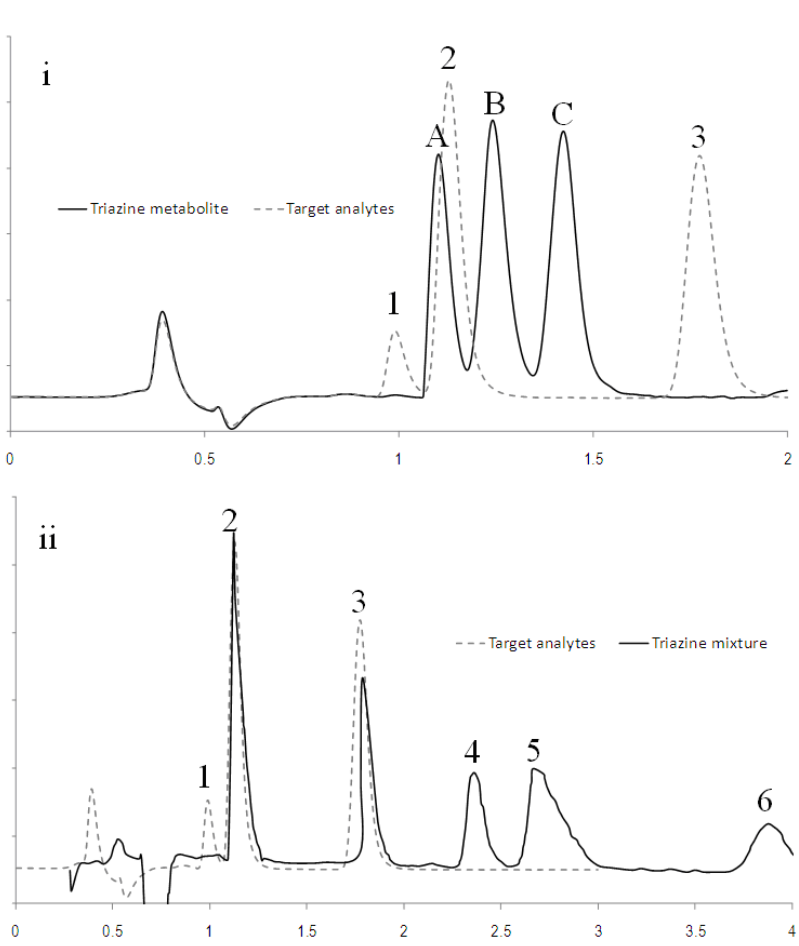



**Figure 5-6: Investigation of potential interference of other triazine pesticides and their metabolites with atrazine.**

***NOTE:*** *peaks identified as (A) atrazine-2-hydroxy; (B) atrazine-desisopropyl; (C) atrazine-desethyl; (1) hexazinone; (2) simazine; (3) atrazine; (4) propazine; (5) ametryn; and (6) prometryne. Mobile phase 30:70 (ACN:$H_2O$) with a UV-vis detector (λ 220 nm), flow rate 3.0 mL $min^{-1}$. Chromatogram (i) triazine metabolites overlayed with hexazinone, simazine and atrazine standard chromatogram as a point of reference (500 µL injection; 100 µg $L^{-1}$ pesticide stock solution); (III) triazine mixture (total of five pesticides) overlayed with chromatogram of target analytes a point of reference (500 µL injection; 100 µg $L^{-1}$ pesticide stock solution). Triazine chromatogram digitised from a paper chromatogram by "GraphClick" (Arizona Software, Switzerland).*

## 5.5. Conclusion

Atrazine, simazine and hexazinone were detected within 2 minutes for each sample by large volume direct injection HPLC with a limit of detection of 0.5, 0.6 and 1.0 µg $L^{-1}$, respectively, in milliQ water without pre-concentration. Validation of the method was performed using a series of analytical standards, with good correlation achieved with samples spiked at 10 and 50 µg $L^{-1}$ for all three pesticides.

Analysis of natural waters comprising various concentrations of natural organic matter (DOM; 3.1 – 11.7 mg $L^{-1}$) had no significant effect on the resolution or separation capacity of the described HPLC method. It was shown that 'like' compounds, such as other triazines and

atrazine metabolites can be differentiated from the target analytes. However, it was found that atrazine-2-hydroxy has the potential to co-elute with simazine under the described operating conditions. Monocrotophos and dicrotophos were deemed unsuitable for analysis by the HPLC system described; both analytes co-eluted with the DOM peak for both the packed and monolithic separation systems.

The use of a monolithic column was demonstrated to significantly decrease the operating pressure of the HPLC system while maintaining the analytical performance of a conventional packed column. While it is acknowledged that the operating pressure using the monolithic column is still considered large for an FIA system at a flow rate of 3.0 mL $min^{-1}$, the use of multiple pumps in series at a reduced flow rate would enable its integration in FICA (this is the focus of Chapter Nine).

# CHAPTER SIX: COMPARISON OF TWO CHEMILUMINESCENT REAGENTS

A major feature of flow injection chemiluminescence is the ability to select a reaction or manipulate parameters to yield a response from a specific analyte within the matrix with relative ease. Wang *et al.* (2001) developed a chemiluminescence method whereby organophosphates could generate chemiluminescence directly when reacted with luminol and $H_2O_2$ in the presence of a sensitizer, making way for a cheaper, simpler alternative to enzymatic chemiluminescence reactions. However, since organophosphate pesticides containing aliphatic amines account for approximately 40% of all organophosphate pesticides, tris(2,2'-bipyridyl)ruthenium(III) could be used as an alternative reagent for these types of pesticides.

Monocrotophos and dicrotophos (Figure 6-1) contain a phosphate moiety, as do all organophosphates. They also have an aliphatic amine moiety, another common functional group in pesticides. The presence of both the phosphate and aliphatic amine functional groups make them ideal pesticides for trials with both the luminol and tris(2,2'-bipyridyl)ruthenium(III) reagents.

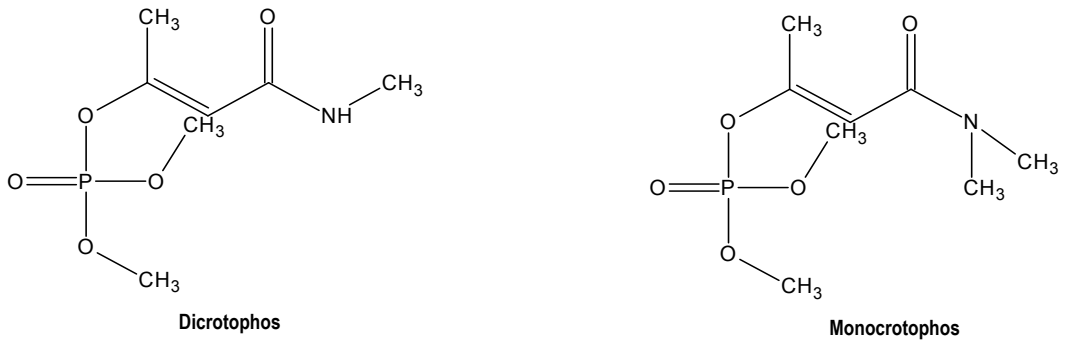



**Figure 6-1: Structure of monocrotophos and dicrotophos**

While the luminol method has been successfully demonstrated using similar organophosphates (*e.g.* dichlorvos, methyl parathion and fenitrothion) (Wang *et al.*, 2001), trials with tris(2,2'-bipyridyl)ruthenium(III) have not. This chapter compares the two reagents and explores the possible interferences.

Previous experience and relevant literature has shown that chemiluminescent reaction conditions vary according to the analyte (Perez-Ruiz *et al.*, 2002; Costin *et al.*, 2003; Perez-Ruiz *et al.*, 2003; Adcock *et al.*, 2004; Costin *et al.*, 2004b; Huertas-Perez *et al.*, 2005). The Minitab 15 statistical program was employed to design experiments to determine the effects of sample/carrier flow rates, reagent concentration, and sample pH. After establishing the best conditions, calibration standards were prepared and analysed. Wang *et al.* (2001) also stated the application of sensitisers in luminol chemiluminescence significantly increased the chemiluminescence response and sensitivity for the determination of organophosphates, thus the effect of sensitisers was also investigated.

A sensitizer in FICA is a chemical compound that transfers energy absorbed from the radiation by the sensitizer to the reacting molecule (*e.g.* chemiluminescent reagent) thus exciting it, and enabling the chemiluminescence to last and be easier to detect (West *et al.*, 1928). Wang *et al.* (2001) determined that from the range of sensitisers studied, 0.1 M cetyltrimethylammonium bromide (CTMAB) was the most effective

## 6.1. Experimental

### 6.1.1. Solution Preparation

All stock solutions, chemicals, reagents and interfering species were prepared as described in Chapter Three. Aqueous solutions (1 mg $L^{-1}$) of monocrotophos and dicrotophos prepared in MilliQ water were used for all optimisation experiments.

### 6.1.2. Tris(2'2-bipyridyl)ruthenium(III) Instrumentation

Tris(2,2'-bipyridyl)ruthenium(II) (15 mM) was synthesised according to Broomhead & Young (1990) (see Section 3.1.1.). All solutions were freshly prepared; the ruthenium complex, prior to injection (100 μL) into a water carrier stream pumped at 6 mL $min^{-1}$, was filtered (0.45 μm syringe filter). The sample stream carrying monocrotophos/dicrotophos (pH adjusted with borax to 9) was pumped at 6 mL $min^{-1}$ (using the FICA instrument previously described, see Figure 4-1).

### 6.1.3. Luminol Instrumentation

The luminol reagent (2.75 mM) was prepared with disodium carbonate, sodium hydrogen carbonate and luminol, in 1.0 L of 0.1 M NaOH solution (see Section 3.1.1.). It was stored at 4°C for 24 hours and returned to room temperature prior to use, to attain stability. Luminol was injected (100 μL) into freshly prepared 0.35 M $H_2O_2$ solution (AR, 30%, Labserv) pumped

at 3 mL $min^{-1}$. The second stream carried monocrotophos/dicrotophos (adjusted to pH 9 with borax) and was pumped at 3 mL $min^{-1}$ (see Figure 4-1).

The points of difference between the two methods and subsequent instrumentation are two fold: Firstly, the reaction mechanism between luminol and organophosphates, as reported by Wang *et al.* (2001) is relatively slow in comparison to tris(2,2'-bipyridyl)ruthenium(III). Luminol chemiluminescence has been observed to occur up to 45 seconds after mixing with organophosphates while with tris(2,2'-bipyridyl)ruthenium(III) it occurs within seconds.

Secondly, due to the slow reaction speed and the reaction being dependent on the oxidation of luminol by peroxide, a mixing coil is used to ensure the reagent is activated (hydrogen peroxide stream and luminol injection) and to maximise the signal generated prior to merging with the sample.

### 6.1.4. Direct Injection HPLC (Method Validation)

The FICA method was validated using the direct injection HPLC method previously described in Chapter Five. The UV–vis detector was set at 220 nm for the analysis of monocrotophos and dicrotophos.

### 6.1.5. GC-FID (Natural Samples Method Validation)

It was anticipated that monocrotophos and dicrotophos could be validated utilising the HPLC validation method described in Chapter Five; however, as illustrated in Chapter Five, the solvent peak and subsequent DOM peak in natural samples co-eluted with both monocrotophos and dicrotophos. Consequently the method validation was performed utilising a GC-FID for natural samples spiked with monocrotophos and dicrotophos.

GC-FID analysis was conducted on a Hewlett Packard 5890. The following operating conditions were applied: Injector temperature 225°C; split valve opened at 0.75 min; split flow 70 mL $min^{-1}$; injection volume 1.0 μL; temperature program: 40°C for 2 min, ramp rate 20°C $min^{-1}$ to 300°C; carrier gas (helium) flow: 33 cm $s^{-1}$ at 37°C; makeup gas (nitrogen) flow: 20 mL $min^{-1}$; column: BPX5, L 30m, (i.d. 0.25 mm, film thickness 0.25 μm). All samples were extracted via SPE, as detailed in Chapter Three.

## 6.2. Results and Discussion

### 6.2.1. Tris(2'2-bipyridyl)ruthenium(III)

#### *Tris(2'2-bipyridyl)ruthenium(III) Optimisation*

Work with ruthenium described in Chapter Four has shown 1 mM tris(2'2-bipyridyl)ruthenium(III) to be the ideal workable concentration, and pH and flow rate to be the main variables that influence the chemiluminescence signal of compounds containing aliphatic amines. However, in preliminary trials with organophosphates, 1 mM tris(2'2-bipyridyl)ruthenium(III) did not produce a significant signal over the pH and flow rate range studied, consequently the concentration was increased from 1 – 20 mM (in increments of 2, 10, 15 and 20 mM). The 15 and 20 mM solutions gave identical responses for a 1 mg $L^{-1}$ dicrotophos solution (pH 10), while 2 and 10 mM gave no significant response. A concentration of 15 mM tris(2'2-bipyridyl)ruthenium(III) was used for subsequent experiments.

The effect of flow rate on chemiluminescence was also studied. A flow rate ranging from 3 mL $min^{-1}$ (generating a signal to blank ratio (SBR) of 1.15 for dicrotophos and 1.35 for monocrotophos) to 9 mL $min^{-1}$ (dicrotophos SBR 1.20 and monocrotophos SBR 1.33) was investigated using 1 mg $L^{-1}$ monocrotophos and dicrotophos at pH 9. The highest dicrotophos SBR (1.45) and monocrotophos SBR (2.20) were obtained for a flow rate of 6 mL $min^{-1}$.

The SBR for a series of 1 mg $L^{-1}$ monocrotophos and dicrotophos solutions, buffered (50 mM phosphate/borax buffer) over the range pH 2-12 was measured giving a maximum at pH 9 (see Figure 6-2).

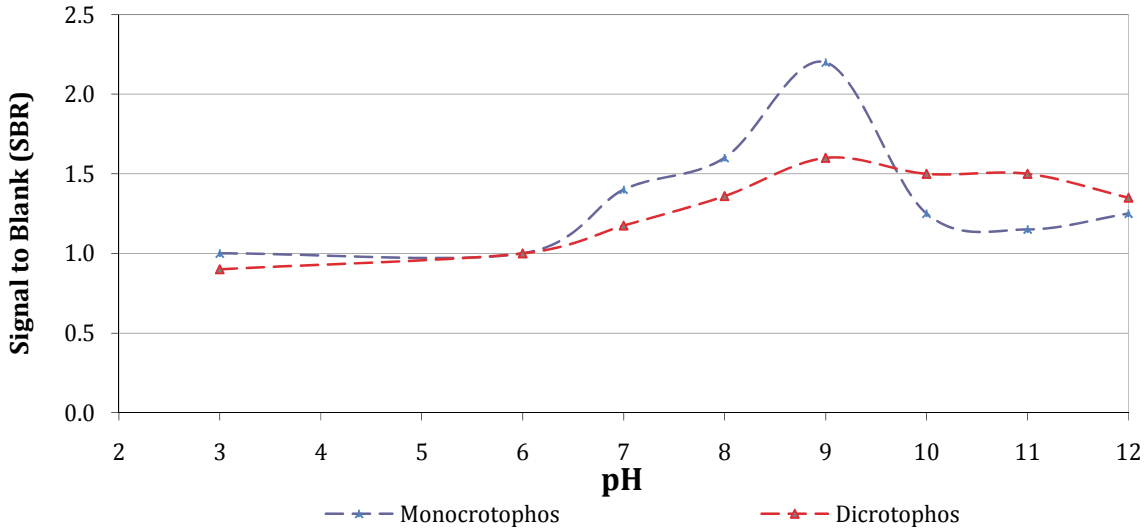



**Figure 6-2: Organophosphate chemiluminescence variation with change in sample pH.**

***NOTE:*** *Signal to blank ratio (SBR) for 1 mg $L^{-1}$ monocrotophos and dicrotophos at various pH with 15 mM tris(2'2-bipyridyl)ruthenium(III) reagent, in a MilliQ water carrier. Carrier and sample flow rate 6 mL $min^{-1}$.*

### *Possible interference*

As described in greater detail in Chapter Seven, tris(2'2-bypridyl)ruthenium(III) chemiluminescence was enhanced in waters containing natural organic matter.

## 6.2.2. Luminol

### *Luminol optimisation*

An experiment was designed using MiniTAB 15 incorporating the low, mid and high settings for luminol (reagent concentration), $H_2O_2$ (concentration), flow rate and sample pH to determine their effects on the intensity of chemiluminescence generated (see Appendix F for more details). The mid point values for luminol (2.75 mM), $H_2O_2$ (0.35 M) and sample pH (7) were selected according to Wang *et al*. (2001) and Gok & Ates (2004), while low and high points were selected based on previous studies (Du et al., 2003; Huertas-Perez *et al.*, 2005; Yaqoob *et al.*, 2004b). The sample values used were pH 2, 7, and 10; sample and carrier flow rate 1.00, 3.00, and 5.00 mL $min^{-1}$; luminol concentration 0.50, 2.75, and 5.00 mM; and hydrogen peroxide concentration 0.20, 0.35, and 0.50 M.

The results (Figure 6-3) showed that the best conditions were similar to those of Wang *et al.* (2001) with the exception of flow rate. The setup implemented by Wang *et al.* was similar in design to the instrumentation designed in this experiment; however, the length of tubing employed by Wang *et al*. was not defined. Wang *et al.* concluded that the best flow rate was 1 mL $min^{-1}$, whereas in this study a flow rate 3.0 mL $min^{-1}$ was best. From the works cited

above it appears that increasing the pH of luminol would be likely to increase the chemiluminescence signal (Wang *et al.*, 2001; Rao *et al.*, 2002; Huertas-Perez *et al.*, 2005). When the effect of pH on luminol was studied with dicrotophos in the range 10-12 (NaOH and sodium carbonate buffer), luminol at pH 12 emitted the most intense response.

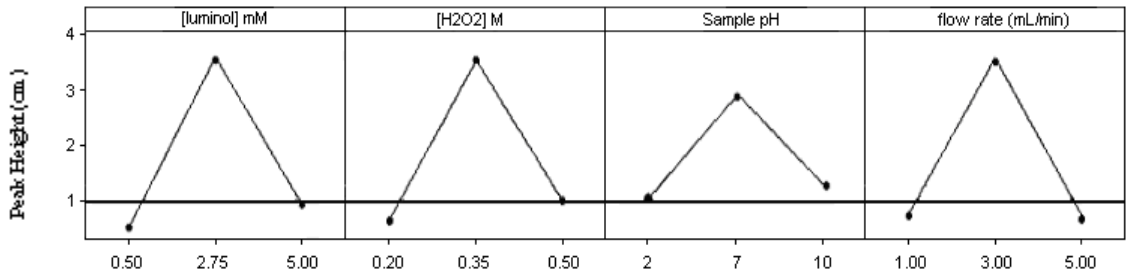



**Figure 6-3: Effect of luminol FICA parameters on peak height.**

***NOTE:*** *Effect of luminol FICA parameters and $H_2O_2$ concentration, sample pH and sample/carrier flow rate on peak height. Luminol for the design experiment was kept constant at pH12.*

### *Luminol sensitiser study*

The effect of CTMAB (Unilab, Australia) on luminol chemiluminescence for the detection of dicrotophos was studied. The addition of NaBr (Unilab, Australia) and NaCl (Unilab, Australia) over the same concentration range was also evaluated (after Wang *et al.,* 2001; see Table 6-1). The sensitisers were added to the sample, the luminol reagent or the hydrogen peroxide solution to determine the best method for addition. It was found that addition of NaBr (3 M) to the sample prior to analysis was the only method that led to signal enhancement. However, it is interesting to note that CTMAB at concentrations greater than 0.05 M formed a slurry and could not be pumped, while CTMAB at 0.05 M generated a signal less than that of the blank, which is a contradiction to the observations reported by Wang.

**Table 6-1: Effect of sensitisers on luminol chemiluminescence**

| Sensitizer [M] | Signal to blank ratio (SBR) | | | |
|---|---|---|---|---|
| | CTMAB | NaBr | NaCl | Water |
| 3 | U | 2.0 | 1.1 | 1.7 |
| 1 | U | 1.1 | 1.0 | 1.7 |
| 0.2 | U | 1.0 | 1.0 | 1.7 |
| 0.1 | U | 1.0 | 1.0 | 1.7 |
| 0.05 | 0.9 | NS | NS | 1.7 |

***Note:*** *Samples comprised of 1 mg $L^{-1}$ monocrotophos/dicrotophos (pH 7). Luminol reagent comprised of 0.35 M $H_2O_2$ and 2.75 mM luminol. NS = No signal obtained. U= un pumpable slurry.*

## 6.2.3. Evaluation of Chemiluminescent Reagents

For 1 mg $L^{-1}$ dicrotophos, SBRs of 2.0 and 1.45 were generated, and for 1 mg $L^{-1}$ monocrotophos, SBRs of 3.12 and 2.25 were generated using the best conditions

determined in this study for the luminol with 3 M NaBr and tris(2'2-bipridyl)ruthenium(III) systems, respectively. Based on these results, luminol with 3 M NaBr as a sensitiser is the better system for the chemiluminescent determination of monocrotophos and dicrotophos in water, and was used for establishing the limit of detection.

## 6.3. Determination of Dicrotophos & Monocrotophos in MilliQ Water

A series of dicrotophos and monocrotophos samples with a concentration range of 0.001-1 mg $L^{-1}$ was prepared in MilliQ water and analysed using luminol with 3 M NaBr. Table 6-2 summarises the analytical and statistical parameters achieved for the determination of monocrotophos and dicrotophos by flow injection chemiluminescence (n=4). The log-log calibration function was Y=mX+c; where Y is the response measured (mV) and X is the concentration in concentration units (µg $L^{-1}$), m is the slope and c is the intercept.

**Table 6-2: Figures of merit**

| Statistical Figures of Merit | Luminol | | HPLC | |
|---|---|---|---|---|
| | monocrotophos | dicrotophos | monocrotophos | dicrotophos |
| Intercept | 1.484 | 0.3043 | 8.8 | 645 |
| Slope | 0.063 | 0.0827 | 12.5 | 8724 |
| Linear dynamic range (µg $L^{-1}$) | 5-100 | 20-100 | 4.5-100 | 0.6-100 |
| LOD[a] (µg $L^{-1}$) | 7.6 | 18.1 | 2.0 | 0.2 |
| LOQ[b] (µg $L^{-1}$) | 17.0 | 135.0 | 8.2 | 0.8 |
| MDL[c] (µg $L^{-1}$) | 20.2 | 15.2 | 1.6 | 1.2 |
| Standard Deviation of the Slope | 0.007 | 0.08 | 0.5 | 246 |
| Standard Deviation of the Intercept | 0.173 | 0.01 | 7.4 | 524 |
| Standard Deviation of the Regression | 0.281 | 0.02 | 11.1 | 719 |
| Correlation Coefficient | 0.9536 | 0.9991 | 0.9949 | 0.9976 |

***NOTE:*** *[a]Limit of Detection calculated using 3 x SD of the blank (n=36 (Harris, 1997). [b]Limit of Quantification calculated using 10 x SD of the blank (n=36) (Wang et al., 2001). [c]Practical Method Detection Limit calculated using lowest standard (n=8), the standard deviation was calculated and multiplied by the one-sided t distribution (Harris, 1997).*

As illustrated in Table 6-2, the limits of detection obtained for monocrotophos and dicrotophos were above that achieved by HPLC. However, the limit of detection obtained for monocrotophos by FICA is similar to that obtained by Du *et al.* (2003). Du *et al.* (2003) employed luminol chemiluminescence for the determination of monocrotophos in water, with a detection limit of 7.0 µg $L^{-1}$; FICA has not previously been employed for the determination of dicrotophos.

It should be noted that neither monocrotophos nor dicrotophos have an ADWG value. However, monocrotophos does have a health trigger valve of 1 µg $L^{-1}$. While the detection

limit of monocrotophos was 7.6 µg $L^{-1}$, which is above the health trigger value, it could be further reduced using online extraction and pre concentration.

MilliQ water spiked with increasing amounts of monocrotophos and dicrotophos (50 and 100 µg $L^{-1}$) was analysed using the flow injection method and the results were confirmed using direct injection HPLC and GC-FID. Table 6-3 reports observed concentrations and recovery for both analytical methods. The recoveries for both methods were within 7%.

**Table 6-3: Recovery for MilliQ water samples spiked with monocrotophos (Mon) and dicrotophos (Dic) using FICA, HPLC and GC-FID (n=4).**

| Analytical method | [Mon] spike (µg $L^{-1}$) | [Mon] observed ± 2sd. (µg $L^{-1}$) | Recovery (%) | [Dic] spike (µg $L^{-1}$) | [Dic] observed ± 2sd. (µg $L^{-1}$) | Recovery (%) |
|---|---|---|---|---|---|---|
| Flow injection | 50 | 53.4 ± 1.5 | 107 | 50 | 48 ± 3.3 | 96 |
| | 100 | 106.2 ± 1.3 | 106 | 100 | 103 ± 3.4 | 103 |
| HPLC direct injection | 50 | 49.6 ± 0.5 | 99 | 50 | 51 ± 3.8 | 102 |
| | 100 | 99.8 ± 0.8 | 100 | 100 | 96 ± 1.7 | 96 |
| GC-FID | 50 | 47.1 ± 2.0 | 94 | 50 | 52 ± 7.2 | 104 |
| | 100 | 95.0 ± 3.6 | 95 | 100 | 102 ± 6.7 | 102 |

### 6.3.1. Possible Interferences

Most environmental samples containing monocrotophos and dicrotophos are also likely to contain DOM. Whilst the interference of some compounds with the chemiluminescence of luminol has been investigated, the effect of DOM has not been documented in the readily available literature. Wang *et al.* (2001) studied the effect of metal ions ($Fe^{3+}$, $Cu^{2+}$ and $Co^{2+}$) and showed that the addition of EDTA reduced the effect of the metal ions. Yaqoob *et al.* (2004a & 2004b) evaluated the effect of silicates and phosphates on luminol chemiluminescence. It was found that they do not readily react with luminol unless the sample is acidic and with the addition of molybdate prior to mixing with luminol (Yaqoob *et al.,* 2004a; 2004b). Borman *et al.* (2009) investigated the effect of DOM on the detection of $Fe^{2+}$ using luminol, it was found that DOM reduced the sensitivity of the chemiluminescence response of iron; however, there was no effect on luminol chemiluminescence for the detection carbaryl in natural waters.

The natural water samples previously presented in Table 5-4 were spiked with 50 µg $L^{-1}$ of monocrotophos and dicrotophos, and analysed using the luminol system with 3 M NaBr as a sensitiser. In this case, DOM had no discernible effect on the chemiluminescent detection of

either monocrotophos or dicrotophos (Table 6-4 summarises the recoveries of the spiked natural water samples).

As previously stated, validation by HPLC was unsuccessful; the DOM present in the natural samples co-eluted with the monocrotophos and dicrotophos peaks. As a result, validation was performed using GC-FID after sample extraction using SPE.

**Table 6-4: Recovery and statistical comparison of luminol FICA and direct injection HPLC for the determination of monocrotophos and dicrotophos (n=3)**

| Sample | Monocrotophos 50 µg $L^{-1}$ (n=3) | | Dicrotophos 50 µg $L^{-1}$ (n=3) | |
|---|---|---|---|---|
| | FIA (RSD%) | GC-FID (RSD%) | FIA (RSD%) | GC-FID (RSD%) |
| MilliQ | 99.6 (3.8) | 101.3 (5.9) | 99.4 (1.8) | 102.9 (4.5) |
| 1 | 97.9 (4.5) | 95.9 (6.8) | 95.4 (3.4) | 98.2 (6.2) |
| 2 | 96.4 (3.1) | 95.5 (7.1) | 98.5 (2.9) | 98.1 (5.1) |
| 3 | 96.2 (3.6) | 97.1 (7.6) | 95.1 (2.7) | 97.4 (6.3) |
| 4 | 97.1 (4.1) | 97.3 (6.4) | 99.6 (2.7) | 99.6 (5.7) |
| 5 | 99.6 (1.9) | 95.6 (8.9) | 99.7 (1.5) | 97.6 (6.8) |
| 6 | 98.9 (2.7) | 96.2 (9.9) | 97.4 (2.8) | 99.7 (4.9) |

## 6.4. Conclusions

FICA with luminol was successfully applied to the detection of dicrotophos (LOD 18.1 µg $L^{-1}$) and monocrotophos (LOD 7.1 µg $L^{-1}$) in MilliQ water and natural water samples containing DOM. Chemiluminescence generated using luminol was found to be better than with tris(2'2-bipridyl)ruthenium(III) for the selected organophosphates because of its greater sensitivity and freedom from interference. While the detection limit was above the current health trigger value set in the ADWG, it could be further reduced using online extraction and pre concentration.

In addition, it was observed that monocrotophos and dicrotophos co-eluted with the DOM present in natural samples using HPLC as the validation method. This suggests that these two pesticides may not be suitable for analysis by FICA using tris(2'2-bipridyl)ruthenium(III)

and a monolithic column (as outlined as a possibility in Chapter Five), as any residual DOM that may be present will result in a false positive for monocrotophos and dicrotophos.

In the following chapters the FICA system presented in Chapter Four will be applied for the determination of atrazine in natural waters. In addition, an investigation on the influence of possible interfering compounds and other pesticides is presented with cross validation using the developed direct injection HPLC method described in Chapter Five.

# CHAPTER SEVEN: APPLICATION OF FICA TO NATURAL WATERS

This Chapter presents the application of the previously described FICA system to natural waters spiked with pesticides, followed by an investigation into possible interferences.

## 7.1. Experimental

All chemicals and reagents used throughout this chapter are presented in Chapter Three.

### 7.1.1. FIA chemiluminescence Instrumentation

The FICA system used and the parameters of operation are described in Chapter Four.

### 7.1.2. Direct Injection HPLC (Validation Method)

The FICA method was validated using the direct injection HPLC method previously described in Chapter Five.

### 7.1.3. Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Water samples (1 L) were filtered under vacuum (Millipore micro/ultrafiltration system) prior to the membranes being analysed (while moist) by ATR-FTIR spectroscopy (Perkin-Elmer Spectrum 2000, Perkin Elmer, USA) as described in Chapter Three.

## 7.2. Results and Discussion

### 7.2.1. Influence of Interfering Species

The influence of the concentration of a selection of cations, anions, natural organic fractions (based on the concentrations likely to be found in natural waters) and selected amine-containing molecules on the analysis of atrazine by FICA was ascertained (Table 7-1). The interference study comprised MilliQ water solutions spiked with 30 µg $L^{-1}$ atrazine and MilliQ water blank solutions (buffered to pH 9 or as stated) spiked with 0.1 to 100 mg $L^{-1}$ of each interfering species investigated (at intervals of 0.1, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg $L^{-1}$).

**Table 7-1: Influence of interfering species**

| Species | FICA mg $L^{-1}$ | ADWG[b] mg $L^{-1}$ | Δ SBR | Species | FICA mg $L^{-1}$ | ADWG[b] mg $L^{-1}$ | Δ SBR |
|---|---|---|---|---|---|---|---|
| $Ni^{2+}$ | 100[a] | - | <1% | $Fe^{2+}$ | 40 | 0.3 | 10% |
| $Ca^{2+}$ | 100[a] | 200 | <1% | $Fe^{3+}$ | 2 | 0.3 | 12% |
| $Zn^{2+}$ | 80 | 3 | <1% | Fulvic Acid* | 2 | - | 15% |
| $Mg^{2+}$ | 60 | 0.1 | <1% | Humic Acid# | 5 | - | 13% |
| $Cu^{2+}$ | 80 | 1 | <1% | Humate* | 5 | - | 12% |
| $Cl^{-}$ | 100[a] | 250 | <1% | Tryptophan | 25 | - | 8% |
| $K^{+}$ | 100[a] | - | <1% | Tyrosine | 5 | - | 11% |
| $CO_3^{2-}$ | 100[a] | 200 | <1% | Nitrate | 100 | 50 | <1% |
| $HCO_3^{-}$ | 100[a] | 200 | <1% | Nitrite | 100 | 3 | <1% |
| $Na^{2+}$ | 80 | 180 | <1% | Ammonia | 0.2 | 0.5 | 25% |
| $Al^{3+}$ | 100[a] | 0.2 | <1% | pH^ | 7-10 | 6.5-8.5 | Exponentially |

***NOTE:*** *Influence of interfering species on the analysis of atrazine by flow injection chemiluminescence, and the subsequent increase/decrease in SBR. (number of replicates, n=4).* [A]*Minimum concentration that caused an increase/decrease in chemiluminescence response (SBR).* [a] *Maximum concentration tested.* [b]*Australian Drinking Water Guidelines (2004) minimum recommended guideline value for physical and chemical characteristics.* [*]*Brown coal origin, north east Victoria.* [#]*Technical grade standard.* [^]*pH adjustments made with the addition of 2M HCl and 2M NaOH.*

There was no significant interference from the metal cations or anions with the exception of $Fe^{3+}$ and $Fe^{2+}$ (2 mg $L^{-1}$ and 40 mg $L^{-1}$ respectively, which are above Australian Drinking Water Guidelines (ADWG)). The DOM fractions (above 5 mg $L^{-1}$ for humic acid and humate and at 2 mg $L^{-1}$ for fulvic acid) produced a positive response, while samples with a pH above pH 9 or an ammonia concentration greater than 0.2 mg $L^{-1}$, increased the blank signal significantly. Tryptophan and tyrosine produced a positive response, the chemiluminescent reaction is due to the two amino acids comprising secondary aliphatic amine functional groups which are known to react with the tris(2,2'bypyridyl)ruthenium (III) reagent (Costins *et al.,* 2004)

### 7.2.2. Atrazine Degradation Products and Other Pesticides

Atrazine is degraded both biologically and photochemically in aqueous environments, with the rate of degradation being dependent upon numerous variables (Ware, 2000). In the described flow injection method, it was expected that the degradation products of atrazine would give a similar chemiluminescence, since the metabolites of atrazine also contain secondary and primary aliphatic amines.

A chemiluminescent response was generated when atrazine metabolites and other triazine pesticides were used in the described system, and although the chemiluminescent intensities were slightly weaker (Figure 7-1), the ability of the system to detect triazines and

their metabolites was demonstrated. The SBRs shown (ranging from 11-14) for triazine pesticides (simazine and hexazinone) and atrazine metabolites (atrazine-2-hydroxy, atrazine-desethyl and atrazine-deisopropyl) were produced using a concentration of 20 µg $L^{-1}$ in MilliQ water with 50 mM borax (pH 9).

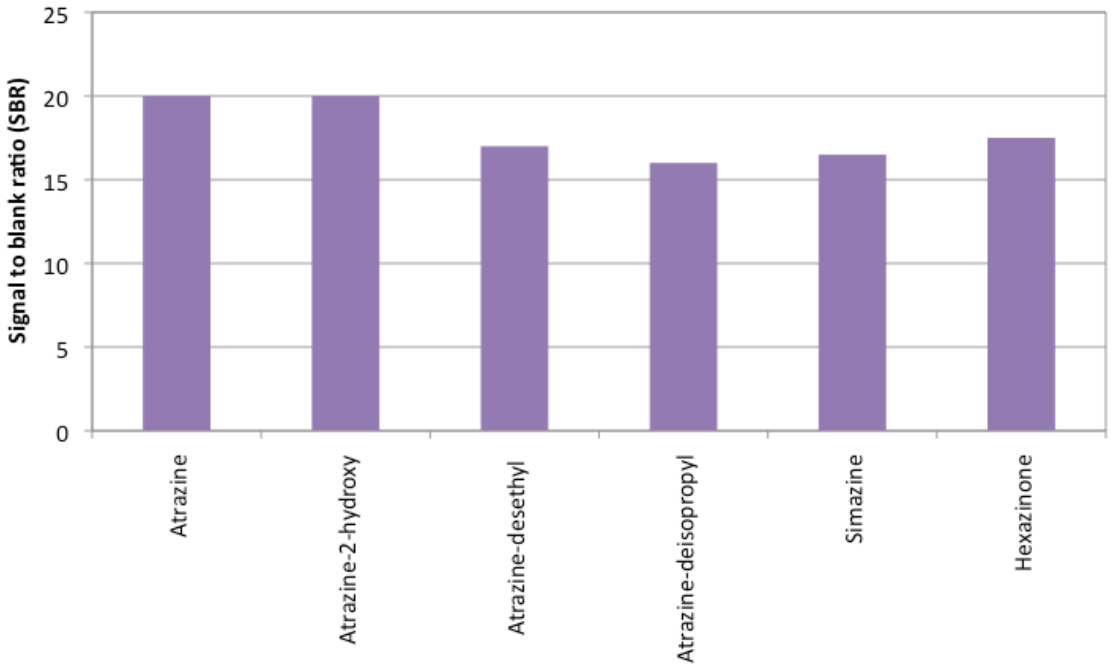



**Figure 7-1: Analytical response from potential FIA chemiluminescence interferences.**

***NOTE:*** *Chemiluminescence response for a range of triazine pesticides (simazine and hexazinone) and atrazine metabolites (atrazine-2-hydroxy, atrazine-desethyl and atrazine-deisopropyl) using a concentration of 20 µg $L^{-1}$ in MilliQ water with 50 mM borax (pH 9) with 1 mM tris(2'2-bipyridyl)ruthenium(III) reagent.*

### 7.2.3. Natural Samples

Natural organic matter originates from decaying plant and animal litter. It is a very complex mixture of compounds containing a wide variety of functional groups, including amines and hydroxyl groups (Lee *et al.*, 2005; Chow *et al.*, 2006). Since amine groups are known to cause a chemiluminescent response with tris(2,2'-bipyridyl)ruthenium(III) (*e.g.* Gerardi *et al.*, 1999, Costins *et al.*, 2004 and Gorman *et al.*, 2006), it was anticipated that DOM would cause interference with the detection of atrazine; but to what extent was unknown.

To test the effect of the presence of DOM on the detection of atrazine, six water samples collected throughout Victoria, Australia, in 2006 (see Table 5-4) were spiked with 10 µg $L^{-1}$ atrazine and adjusted to pH 9 (50 mM borax) prior to testing.

### 7.2.4. DOM Interference

The signal from the chemiluminescence emitted in natural water samples was significantly greater than for the control sample (10 µg $L^{-1}$ atrazine in MilliQ water pH 9, SBR 8.20), masking the signal generated from reaction of atrazine with 1 mM tris(2'2-bipyridyl)ruthenium (III) (Figure 7-2). It was originally thought that by spiking the natural water sample with a large dose of atrazine that either the emitted signal would be larger than the natural water blank, or a significant change in the peak profile might occur as a result of a difference in the kinetics of the production of chemiluminescence. However, close inspection showed no changes in the peak characteristics, and the interfering signal completely dominated the signal from atrazine.

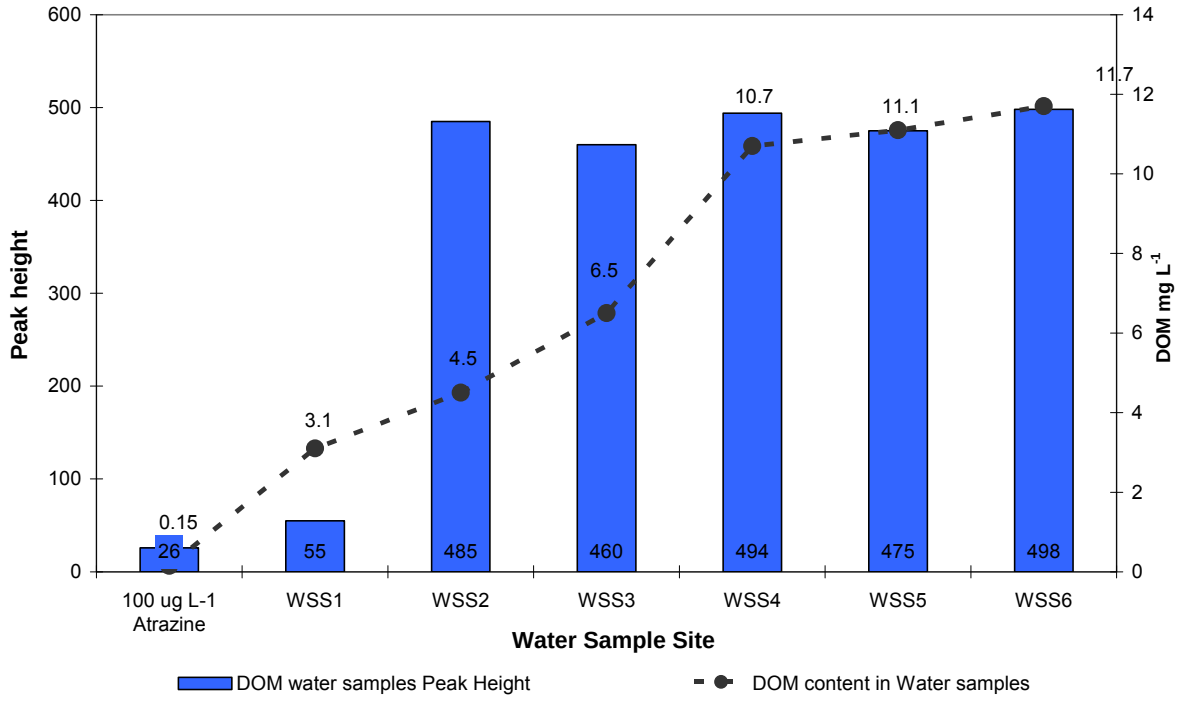



**Figure 7-2: Chemiluminescence response atrazine spiked natural samples**
***NOTE:*** *all six natural water samples were spiked with 10 $L^{-1}$ atrazine and buffered with 50 mM Borax to pH 9. The generated signal for six natural waters far exceeds signal generated from the clean standard.*

### 7.2.5. DOM Interference Investigation

To confirm the identity of the interference, a 1 L aliquot of each of the natural water samples was filtered first through a microfiltration membrane (0.22 µm) and then through an ultrafiltration membrane (100 kDa). Microfiltration did not remove the interference but ultrafiltration reduced the intensity. This showed that some of the interfering molecules or colloidal particles were less than 0.22 µm but greater than 100 kDa. ATR-FTIR spectra of the fouled ultrafiltration membrane showed the presence of amine and hydroxyl functional

groups. The spectra provided strong evidence that the cause of the interference was due to DOM with distinctive peaks present at 1500 – 1650 $cm^{-1}$ (amine bend); 1200 – 1500 $cm^{-1}$ (hydroxyl bend) and 950 – 1300 $cm^{-1}$ (amine stretch) above the blank non fouled membrane (see Figure 7-3).

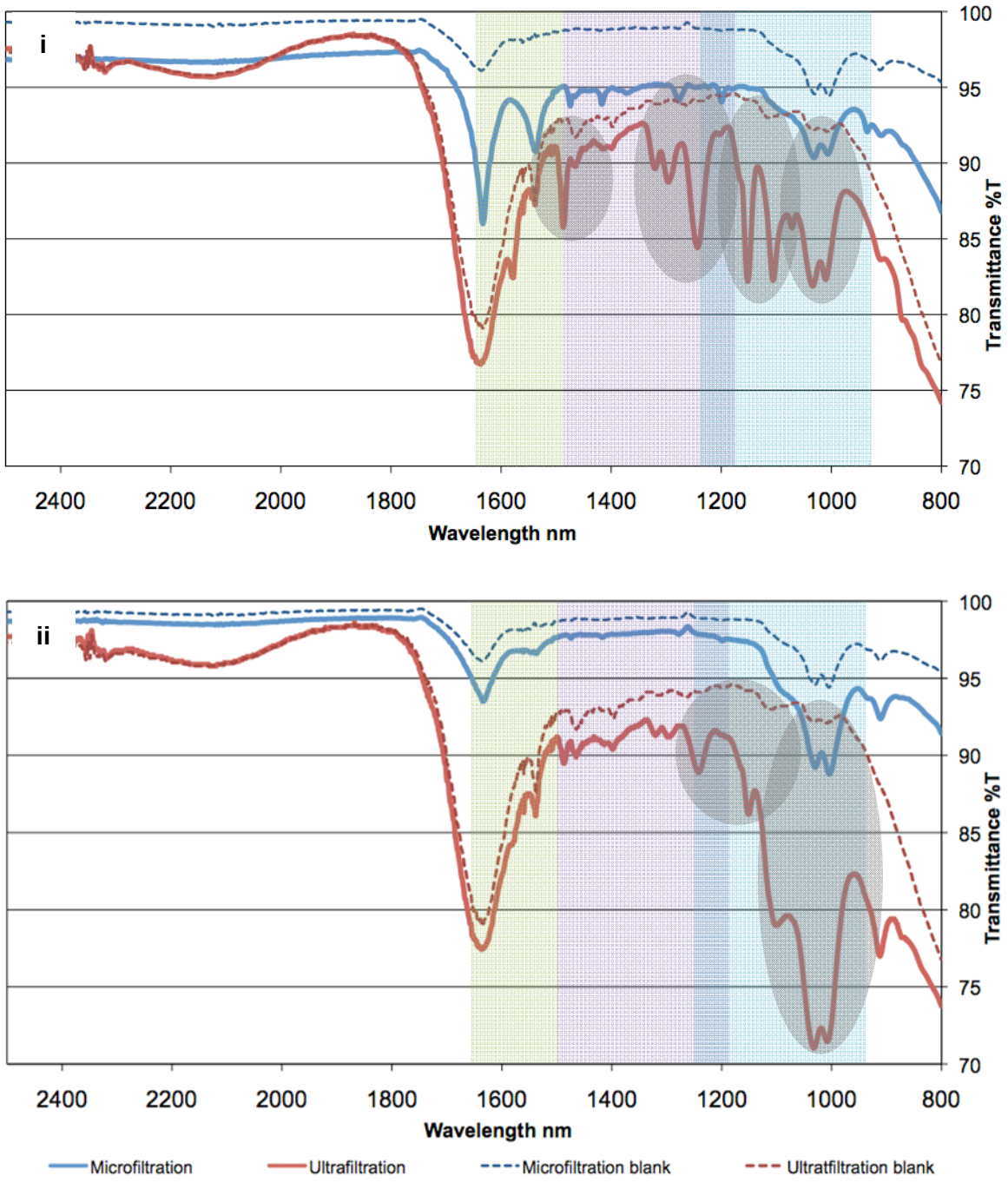



**Figure 7-3: ATR-FTIR spectroscopy of natural water samples.**

***NOTE:*** *(i) sample number two; and, (III) sample number six. Spectra clearly indicate the presence of amine and hydroxyl functional groups which are not removed by filtering during sample microfiltration prior to analysis, but are retained on the filtering membrane during ultrafiltration. The spectra provide an explanation as to a possible cause of interference from the natural DOM with distinctive peaks present at 1500 – 1650 nm (amine bend); 1200 – 1500 nm (hydroxyl bend) and 950 – 1300 (amine stretch) above the background noise from the filtration membrane (blank). See Appendix E for ATR-FTIR spectra for each water sample.*

## 7.3. Removal of DOM

The interference caused by DOM in natural samples in the determination of atrazine was overcome through solid phase extraction (see Section 3.3). It is well documented that C18 SPE can be used to capture atrazine from natural waters with recoveries as high as 95%, depending on the nature and retention of the natural organic matter (JunkerBuchheit & Witzenbacher, 1996; Zambonin & Palmisano, 2000; Trajkovska *et al.*, 2001). The six natural water samples (see Table 5.3) and a MilliQ water sample (1 L, n = 9) were filtered (0.45 µm nylon filter, Millipore), spiked with 0.5 µg $L^{-1}$ (n = 3) or 10 µg $L^{-1}$ (n = 3) atrazine and passed through an SPE cartridge. Table 6-2 reports the recoveries and statistical method comparison of atrazine from these samples. A paired t-test was performed to determine if the FICA and HPLC methods strongly correlated. The mean difference between the methods (0.5 µg $L^{-1}$; M=0.006, SD =0.02, N= 7 and 10 µg $L^{-1}$; M=-0.3, SD =0.35, N= 7) was not significantly greater than zero, t-test (6) = 0.39 and -0.74 respectively, two-tail p = 0.71 and 0.49 respectively, providing evidence that the two methods strongly correlate.

The addition of the SPE step removed the interference from DOM (since it was not retained on the cartridge), and concentrated the sample, greatly enhancing sensitivity. The detection limit was reduced to 14 ng $L^{-1}$ and the recoveries were very similar to those for HPLC. The organic solvent present in the SPE extract enhanced the chemiluminescence response by *ca.* 20%; this is supported in the literature. Lee & Nieman (1996) reported that the use of organic solvent increased the chemiluminescence intensity of tris(2,2'-bipyridyl)ruthenium(III) for proline and oxalate determination. In comparison to other FIA methods described in Table 2-5, the LOD obtained is lower than most methods presented utilising electrochemistry, potentiometric detection, UV-Vis detection and immunoassay fluorescence. However, immunoassay chemiluminescence methods, such as those described by Tudorache *et al*. (2008) (LOD of 0.003 µg $L^{-1}$), were found to be more sensitive. This could be due to the fact that immunoassay methods concentrate the target analyte at the site of detection in contrast to the off-line SPE procedure described in this chapter.

HPLC analysis of the natural water sample extracts revealed that the apparent large recovery observed for the flow injection method for natural water 6 was due to the presence of an unknown compound which caused a chemiluminescent reaction with tris(2,2'-bipyridyl)ruthenium(III). On identifying chemicals used within the sample location, it was found that the sample was contaminated with hexazinone, a triazinone herbicide.

**Table 7-2: Recovery and statistical comparison of FICA and direct injection HPLC**

| Sample | Atrazine 0.5 µg $L^{-1}$ (n=3) | | Atrazine 10 µg $L^{-1}$ (n=3) | |
|---|---|---|---|---|
| | FIA Recovery % (RSD%) | HPLC Recovery % (RSD%) | FIA Recovery % (RSD%) | HPLC Recovery % (RSD%) |
| MilliQ | 97.5 (0.3) | 99.1 (0.5) | 99.3 (0.3) | 98.6 (0.2) |
| 1 | 91.6 (3.8) | 99.1 (0.6) | 83.9 (3.6) | 101.5 (0.5) |
| 2 | 94.9 (0.7) | 94.3 (1.4) | 88.9 (3.5) | 95.5 (0.5) |
| 3 | 86.6 (1.1) | 88.9 (1.5) | 85.7 (0.5) | 89.7 (4.3) |
| 4 | 89.5 (5.2) | 87.6 (2.8) | 92.9 (1.0) | 96.5 (0.7) |
| 5 | 87.8 (6.1) | 90.0 (2.1) | 96.4 (0.5) | 96.7 (1.4) |
| 6 | 114.9 (6.2) | 94.8 (2.4) | 106.8 (6.0) | 93.5 (1.2) |
| **Statistical Comparison - Method Validation** | | | | |
| Correlation coefficient | | | 0.9906 | |
| t-test (6) | 0.39 | | -0.74 | |
| P two tailed | 0.71 | | 0.48 | |

***Note:*** *Recovery and statistical comparison between the validation method and the proposed flow injection method for atrazine from spiked natural water samples and MilliQ water samples using SPE with flow injection chemiluminescence analysis and direct injection HPLC analysis (n=3).*

## 7.4. Conclusions

In Chapter Four, atrazine was detected by FICA using tris(2,2_-bipyridyl)ruthenium(III) chemiluminescence with a limit of detection of 1.3 ± 0.1 µg $L^{-1}$ in MilliQ water without pre concentration. Validation of the method was performed by direct injection HPLC, with a good correspondence between the methods.

However, when applied to natural waters, the presence of DOM causes a significant positive chemiluminescent response which masks the signal from the atrazine. The functional groups responsible for the interference were identified by ATR-FTIR as amines and hydroxyl groups present in the natural water.

When cations and anions were present at levels common in natural waters, only $Fe^{3+}$ and $Fe^{2+}$ (which were at above the ADWG levels) caused interference. It was also shown that

similar compounds, such as the atrazine metabolites and other triazine pesticides, produced a chemiluminescent signal with tris(2,2-bipyridyl)ruthenium(III).

The interference from DOM was removed by SPE (10.0 mL concentrated sample from a 1 L extraction using sample SPE C18 extraction) as the DOM is not retained on the SPE cartridge. This also significantly improved the detection limit for atrazine in natural water samples to 14 ± 2 ng $L^{-1}$ with strong correlation with the HPLC method ($R^2$ = 0.9906, t-test (6) = 0.39 (p two tailed = 0.71) and -0.74 (p two tailed = 0.48) for 0.5 and 10 µg $L^{-1}$ atrazine respectively).

In the following chapter, the incorporation of an in-line SPE manifold will be introduced for the analysis of atrazine, simazine, and hexazinone in natural waters using FICA. While SPE has proven to be successful in eliminating the interference of DOM, it is a time consuming process undertaken offline. In addition, by utilising an in-line SPE manifold, a selection SPE resins can be evaluated to optimise the extraction procedure.

# CHAPTER EIGHT: FICA WITH IN-LINE SOLID PHASE EXTRACTION

The determination of pesticide residues in MilliQ water using tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence was demonstrated (Chapter Four). It was shown that the presence of DOM in natural waters presents a problem, as it masks the chemiluminescence signal generated from the target analyte with tris(2,2'-bipyridyl)ruthenium(III) (Chapter Seven). As previously stated, DOM contains a range of humic and fulvic acids which also have reactive amine groups which can explain the positive interference observed. To overcome this, off-line SPE was employed and detection limits below drinking water guidelines were achieved (Chapter Seven); however, the time required to undertake the SPE extractions and the volume of sample required was significant – characteristics that are not desirable in a portable instrument, such as the one being developed.

This chapter describes the integration of an in-line SPE manifold for the determination of two triazine and one triazinone herbicides (atrazine, simazine and hexazinone) in natural waters. While numerous authors have employed in-line SPE extraction in flow injection systems (see the review by Wang et al, 2009 and Chapter Two), the majority utilized immunoassay techniques as the pre-concentration step. However, the consensus amongst authors was: in-line SPE enabled FIA analysis to be performed with the minimum amount of sample and solvent, while obtaining comparable detection limits to the 'more traditional' off-line extractions (Wang *et al.*, 2009b).

Hence, this chapter describes the evaluation and application of three different SPE resins in order to overcome the inference from DOM, and includes an assessment of their suitability for incorporation as an in-line SPE FICA procedure. One resin was selected for the removal of DOM, and the remaining two SPE resins were used as an analyte trapping sorbent. Confirmation and validation of the methods developed were performed using direct injection HPLC and GC-MS.

## 8.1. Experimental

### 8.1.1. Solution Preparation

All stock solutions, chemicals and reagents were prepared as described in Chapter Three, unless otherwise stated. All natural water samples were filtered through a 0.45 µm nylon filter under vacuum (Millipore, Australia) prior to analysis.

### 8.1.2. Solid Phase Extraction Resins

#### *Resin One: MIEX© Resin*

A 10 g sample of MIEX© (Magnetic Ion Exchange) resin (Orica Pty Ltd Australia, particle size 100- 300 µm) was cleaned with three washings of 100 mL 12% NaCl solution before packing into a micro flow injection extraction column (20.0 mm (L), 170 µL internal volume; GlobalFIA, USA). Prior to analysis, the column was flushed with 100 mL of MilliQ water.

#### *Resin Two: C-18*

A 100 mg sample of Perisorb C-18 resin (Merck, Germany, particle size 30-40 µm) was cleaned with three washings of 25 mL methanol (HPLC grade) prior to packing into micro flow injection extraction column (20.0 mm (L), 170 µL internal volume; GlobalFIA, USA) as a methanol slurry. Before analysis, the column was flushed with four injections (100 µL) of solvent and four injections of MilliQ water to activate and condition the column.

#### *Resin Three: Nexus©*

A 100 mg sample of Nexus© bulk resin (a modified C-18 resin; Varian, USA, particle size of 80-100 µm) was cleaned with three washings of methanol (HPLC grade) prior to packing into micro flow injection extraction column as methanol slurry. Before analysis, the column was flushed with four injections (100 µL) of solvent and four injections of MilliQ water; it is important to note Nexus resin does not require conditioning or activation prior to use, rinsing the column with injections of solvent and water was done as precautionary step between sample injections.

### 8.1.3. FICA with DOM trapping using MIEX© Resin

A flow injection system was set up as shown in Figure 8-2. Both the sample and buffer streams, along with the carrier stream were propelled using two pumps (MilliGat, Global FIA, USA) through bridged PVC and PTFE tubing (1.85/0.76 mm i.d; Global FIA, USA) at 4.6 mL $min^{-1}$. All other tubing was PTFE (0.76 mm i.d; Global FIA, USA). The sample stream was

passed though an extraction column (Figure 8-1 illustrates a schematic of the SPE cartridge packed with MIEX© resin) to remove the DOM from natural samples. The system used is similar to the FIA system described in Chapters Three and Four, however, in this system the sample and buffer streams were merged at a T-piece before passing through a 10 cm mixing coil to fully mix the two solutions. The chemiluminescent reagent (tris(2,2'-bipyridyl)ruthenium(III)) was then injected into the carrier stream and introduced to the buffered sample at a second T-piece prior to the detector (as described in previous chapters; see Figure 8-2 for a schematic of the modified FICA system designed for DOM trapping). *Note: the in-line SPE cartridge is tapered at one end. This is to focus the sample when eluted off the SPE resin.*

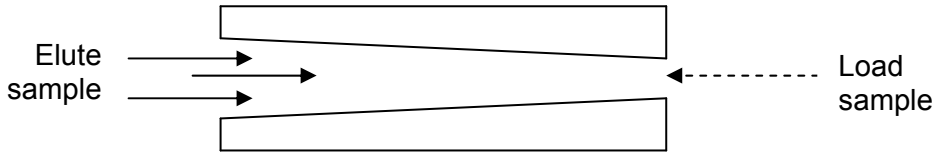



**Figure 8-1: FIA in-line SPE cartridge schematic.**

***Note:*** *after each in-line extraction, the resin was washed thoroughly with solvent prior to re-use. The SPE resin was replaced after six extractions.*

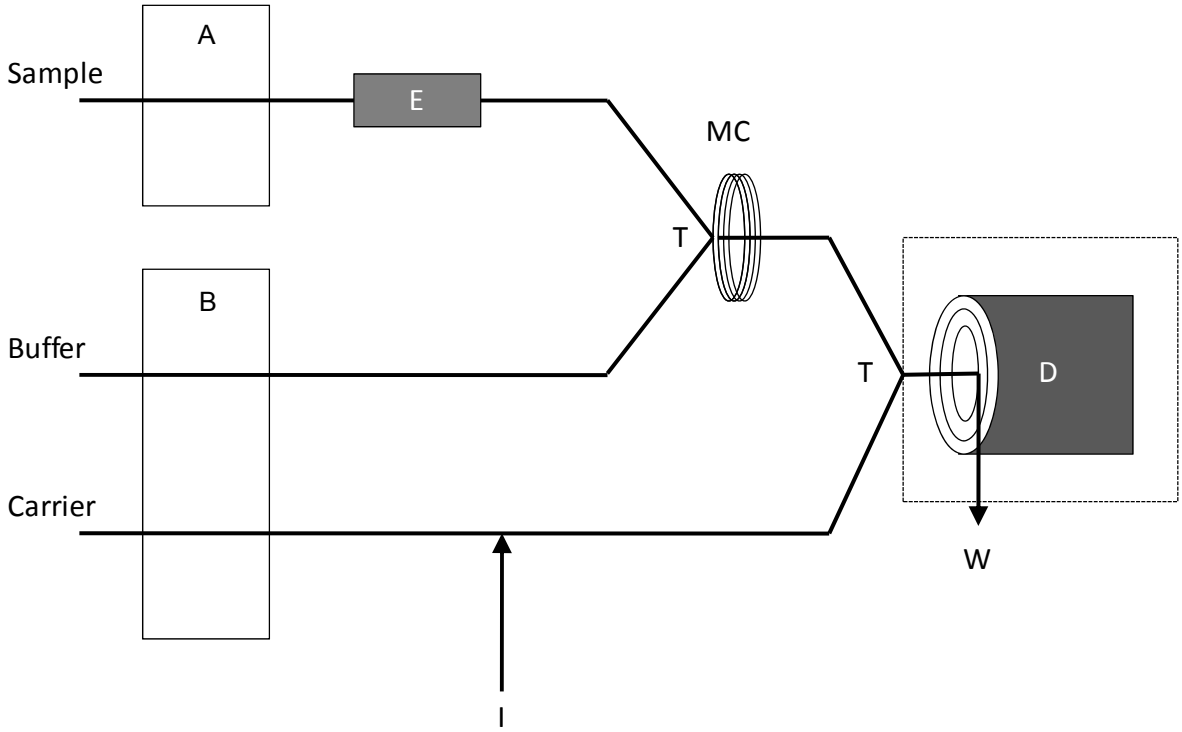



**Figure 8-2: FICA with in line SPE packed with MIEX© for the trapping of dissolved organic matter in natural waters for the determination of pesticides in natural waters.**

***Note:*** *(A) Carrier stream peristaltic pump; (B) Buffer/Carrier stream pump; (I) Injection valve (100 µL 1 mM tris(2,2'-bipyridyl)ruthenium(III)); (E) Extraction column; (MC) mixing coil; (T) T-piece; (D) PMT; and (W) Waste.*

As illustrated in Figure 8-2, the sample and buffer stream are merged after the sample has passed through the extraction column. This is to ensure the sample pH is kept low, enabling the MIEX© resin to remain activated.

### 8.1.4. FICA with Analyte Pre-Concentration using C-18 and Nexus© Resin

A 100 mL of sample (0.45 µm filtered) was propelled using a pump (MilliGat, Global FIA, USA) through bridged PVC/PFTE tubing (1.85/0.76 mm i.d; Global FIA, USA) at 4.6 mL $min^{-1}$ through an extraction column (20.0 mm (L) with titanium frits, 170 µL internal volume; Global FIA, USA). The extraction column was packed with either C-18 or Nexus© for analyte isolation. Organic solvent (100 µL) was injected into the sample stream via a six-port injection valve (Rheodyne, USA) eluting the concentrated analyte, which merged with the buffer stream and was dispersed by a mixing coil. The concentrated sample then mixed with the chemiluminescence reagent which upon mixing produced light detected by the PMT (as previously described; see Figure 8-3 for a schematic of the modified FICA system designed for analyte pre-concentration).

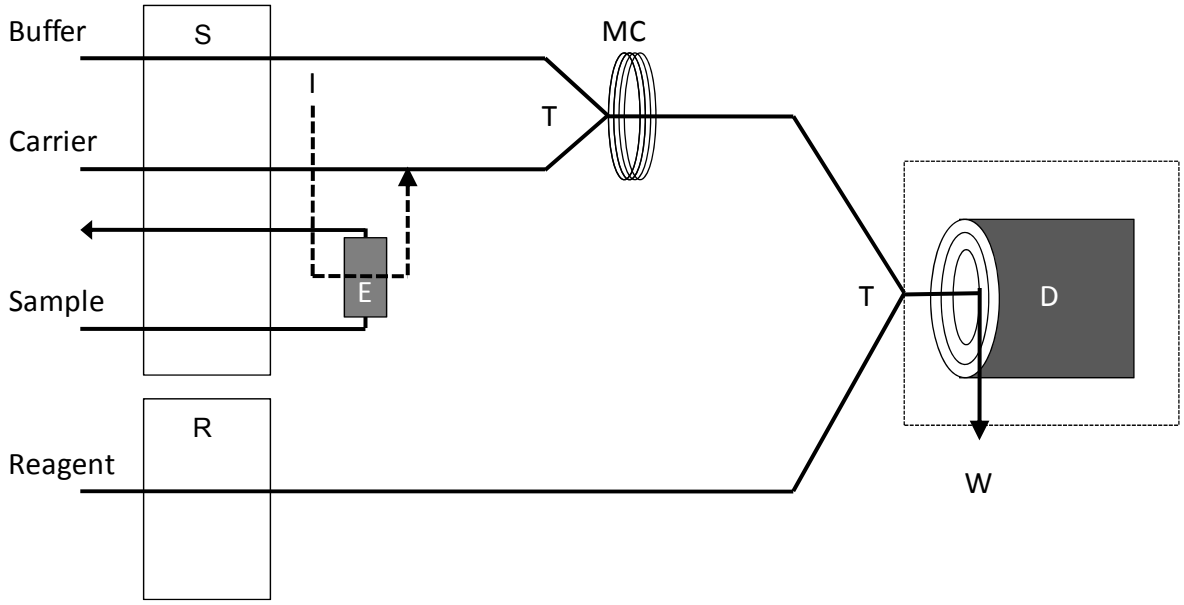



**Figure 8-3: FICA with in line SPE packed with C-18 or Nexus© for the pre concentration and determination of pesticide residue in natural waters.**

***Note:*** *(R) Reagent stream pump (2 mM tris(2,2'-bipyridyl)ruthenium(III) in 20 mM $H_2SO_4$) pump; (S) Sample stream pump; (I) Injection valve (100 µL organic solvent (acetonitrile)); (E) Extraction column; (MC) Mixing coil; (T) T-piece; (D) PMT; and (W) Waste.*

### 8.1.5. Direct Injection HPLC (Sample Validation)

The FICA method was validated using the direct injection HPLC method previously described in Chapter Five.

### 8.1.6. Gas Chromatography – Mass Spectrometry

GC-MS was used to measure the adsorption capacity of the resins according to the method described in Chapter Three (section 3.4.5).

## 8.2. Results and Discussion

### 8.2.1. SPE Characteristics

The incorporation of the SPE materials was to achieve either of the following two tasks: removal of DOM from the sample matrix using MIEX© resin and so eliminating the positive chemiluminescence interference from DOM, or trapping the target analyte from the sample matrix using the C-18 or Nexus© resin and so allowing pre-concentration and lower detection limits (as well as removing the analyte from the DOM sample matrix).

MIEX© resin was developed for the removal of natural organic matter in water treatment (Orica, 2010). It is a micro sized, macroporous magnetic ion exchange resin that enables the resin to regenerate. Due its ability to rapidly extract DOM from water and easily be regenerated (trapped DOM is removed from the resin via flushing with 12% NaCl, enabling the resin to be re-used), MIEX© was selected as a resin for incorporating into the developed FICA system for DOM removal. It was envisaged that the MIEX© resin could be used in-line as a pseudo filter, passing natural samples through the resin at a suitable flow rate to ensure maximum contact time to remove (extract) any DOM present while allowing the analyte(s) of interest (i.e., atrazine, simazine and hexazinone) to pass through to the detector (after reacting with the chemiluminescent reagent).

In contrast to MIEX©, the use of the C-18 and modified C-18 (Nexus©) resins was designed to trap analytes of interest from a complex sample matrix. The C-18 resin selected was chosen to be representative of the typical resins found within SPE cartridges (*e.g.* as a substitute for the SPE cartridges used in Chapter Six). On the other hand, Nexus© is a modified polymeric C-18 resin the use of which was not only designed to capture analytes of interest but also to minimise the steps involved in typical SPE extractions. Nexus© does not require activation or conditioning prior to use (and as a result uses less solvent); in addition, Nexus© has a larger adsorption capacity and working pH range compared to conventional resins (pH range 2 - 12; Varian, 2010). As such, it was envisaged that the C-18 would 'work' as an equivalent conventional SPE extraction resin for analyte pre-concentration to which the Nexus© resin could be compared.

An experiment in which MIEX© resin was used in batch mode was performed (that is, a series of beaker experiments comprising 100.0 mL of MIEX© in a slurry); results obtained indicate that the MIEX© effectively removes both DOM and any pesticide present (confirmed using GC-MS), eliminating MIEX© as a suitable resin for this application.

In addition, scanning electron microscopy (SEM) micrographs were taken to qualitatively assess the resins exposed to natural water (DOM ca. 10 mg $L^{-1}$) and atrazine (100 µg $L^{-1}$ in MilliQ water) during the batch trials. Figure 8-4 illustrates micrographs from the Nexus© resin; Figure 8-5 the C-18 resin; and Figure 8-6 MIEX©.

### 8.2.2. Analytical Comparison of SPE Resins

Table 8-1 summarises the results obtained for 100 mL samples containing atrazine, hexazinone, or simazine ranging in concentration from 1 ng $L^{-1}$ to 10 000 ng $L^{-1}$ in MilliQ water. The samples were analysed using the three SPE materials described, with thorough washing of the resins conducted between each analysis. For illustrative purposes, Figure 8-7 presents an example trace utilizing the Nexus© resin for the determination of atrazine in MilliQ water. As an side, the chemiluminescence signal was enhanced by 27% when acetonitrile was used as the eluent in the C-18 and Nexus© resins.

**Table 8-1: Method detection limits (MDL) for MIEX©, C-18 and Nexus© resins with tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence for 100 mL samples in MilliQ water.**

| Analyte | SPE Resins | | | ADWG[a] |
|---|---|---|---|---|
| | MIEX | C-18 | Nexus | |
| | Method Detection Limit (MDL) ng/L | | | ng/L |
| Atrazine | 2 000 ± 200 | 170 ± 25 | 14 ± 1 | 100 (40) |
| Hexazinone | 8 000 ± 1 600 | 340 ± 40 | 48 ± 6 | 2 000 (300) |
| Simazine | 18 000 ± 2 000 | 275 ± 20 | 32 ± 3 | 500 (20) |

***Note:*** *[a]Australian Drinking Water Guidelines (2004) recommended limit of detection (and health value based on 10% of allowable daily intake)*

The method detection limits for the three resins were above Australian Drinking Water Guideline (ADWG) values, approximately equal to the ADWG values and below ADWG values for MIEX©, C-18 and Nexus© respectively. The MIEX© resin in the SPE cartdrige made no difference to the performance of the system (the mass of MIEX© was later shown to be insufficient to remove the pesticide from solution at a concentration that altered the observed signal (Table 8-2)). While it was thought that the C-18 and Nexus© resins would behave in a similar fashion, there was a significant difference in the method detection limit (MDL), with the Nexus© resin providing the better performance.

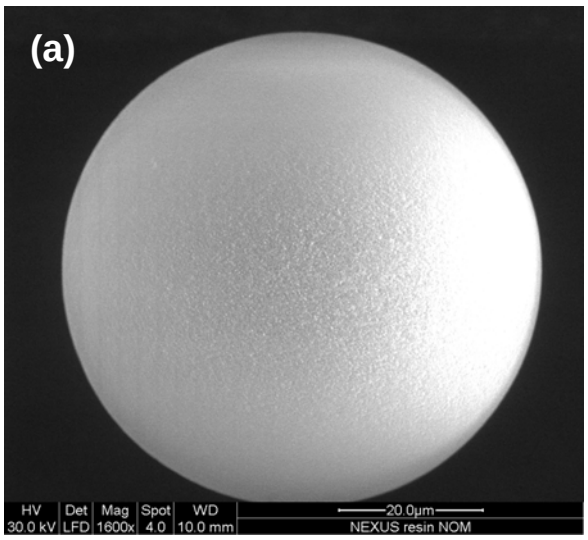
The surface area of the resins was measured by BET isotherms and ultimately the efficiency and suitability of the resins for in-line SPE flow injection chemiluminescence was established (Table 8-2).

**Table 8-2: BET Isotherm analysis for MIEX©, C-18 and Nexus© resins, and the calculated column equivalence volume.**

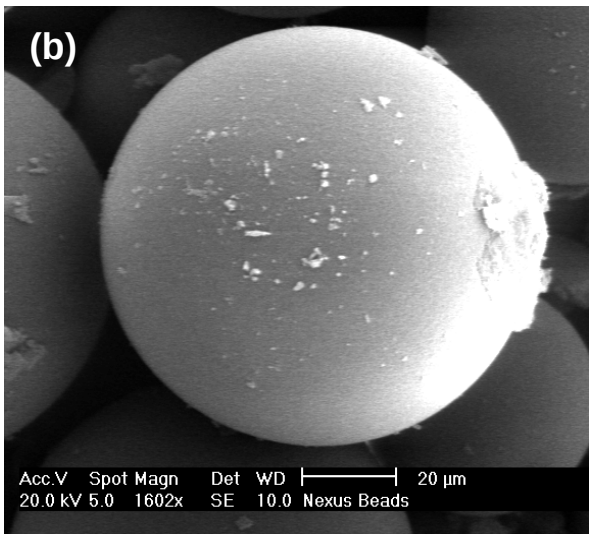
| SPE Resin | Surface Area $m^2/g$ | Surface Area ($m^2$) in FIA column | Surface Area Eq. Ratio* | Mass of Resin Eq. (g)^ | Column Volume (mL)# |
|---|---|---|---|---|---|
| MIEX | 5.78 | 0.34 | 731.62 | 42.87 | 124.38 |
| C-18 | 5.48 | 1.25 | 198.50 | 45.22 | 33.75 |
| NEXUS | 619.52 | 247.81 | 1.00 | 0.10 | 0.17 |

***Note:** * The surface area equivalence is a calculated ratio using the BET isotherm surface area data to standardize the three resins in terms of their determined isotherm surface area.; ^ The mass of resin equivalence is the calculated mass needed for each resin to have equal surface areas; and, # is the calculated column volume needed in order to have all resins of equal surface area.*

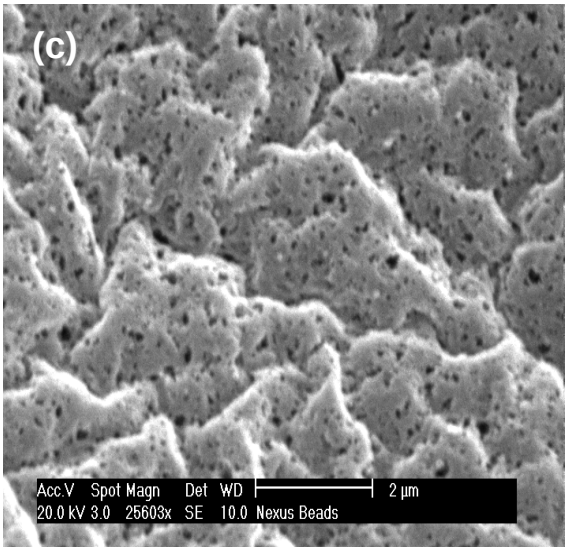
From the data generated for Table 8-2, it cab be seen that the mass of resin required in the in-line SPE extraction column for either the MIEX© or C-18 resins to have an equal surface area to that of the Nexus© are beyond the physical capabilities of a flow injection instrument. Typically, flow injection utilises low pressure pumps with little or no back pressure. For the solutions to be pumped through a 124.38 mL (MIEX© equivalent column volume) or a 33.75 mL (C-18 equivalent column volume) an HPLC pump would be required, operated with a slow flow rate and under a large back pressure.



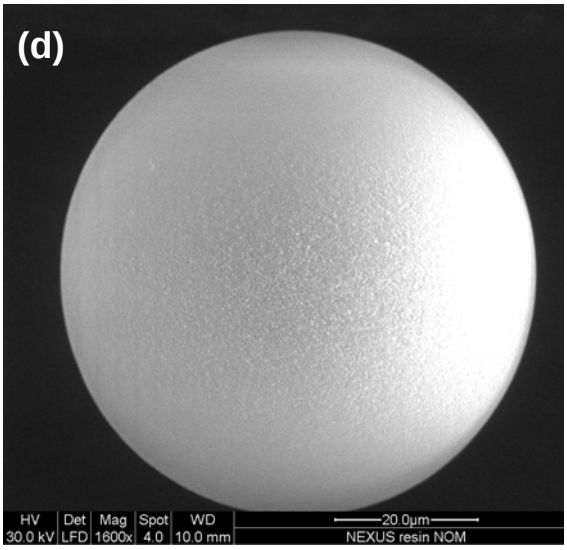

(a) Nexus© resin after exposure to MilliQ water




(b) Nexus© resin after exposure to atrazine solution (100 µg $L^{-1}$ in MilliQ water)

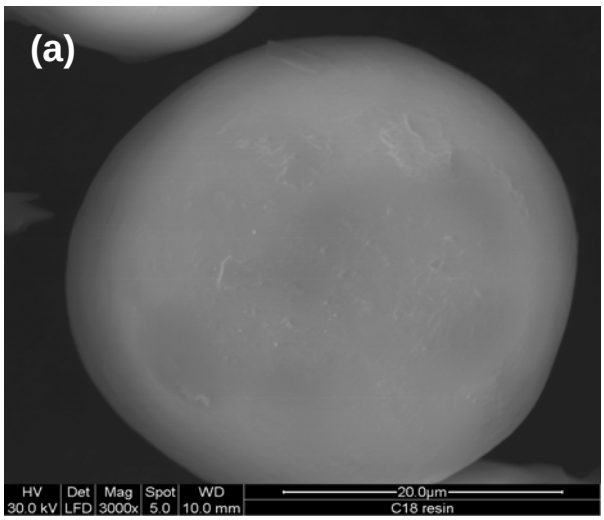



(c) Nexus© resin surface structure

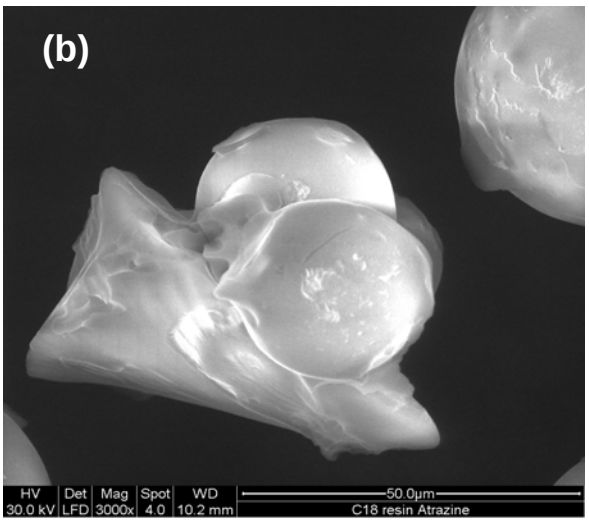



(d) Nexus© resin after exposure to natural water (DOM *ca.* 10 mg $L^{-1}$)

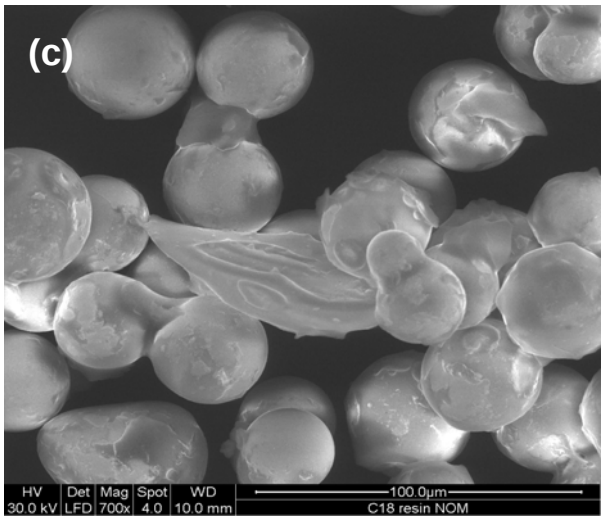
**Figure 8-4: SEM micrographs of Nexus© resin after exposure to natural water and atrazine in MilliQ water.**



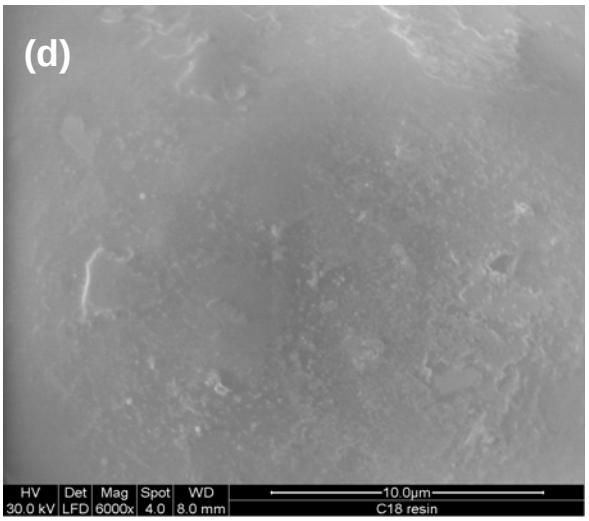

(b) C-18 resin after exposure to MilliQ water




(b) C-18 resin after exposure to atrazine solution (100 µg $L^{-1}$ in MilliQ water)

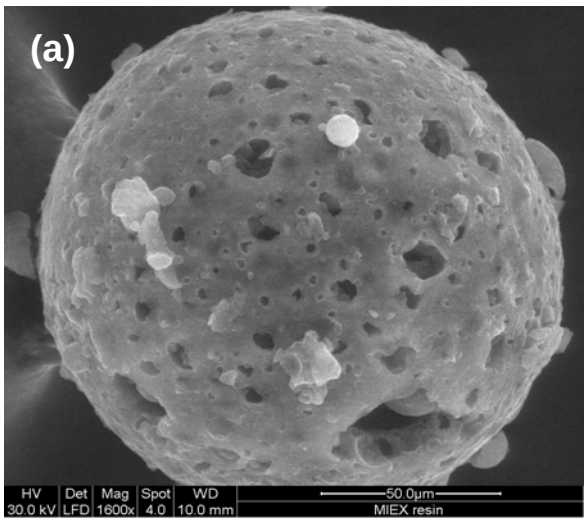



(c) C-18 resin after exposure to natural water (DOM *ca.* 10 mg $L^{-1}$)

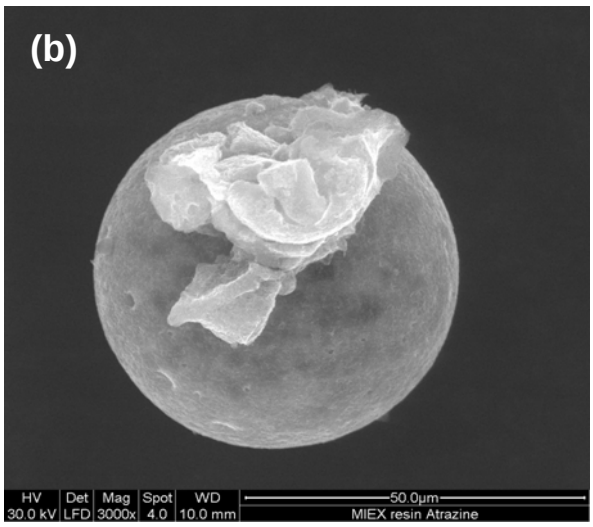



(d) C-18 resin surface structure

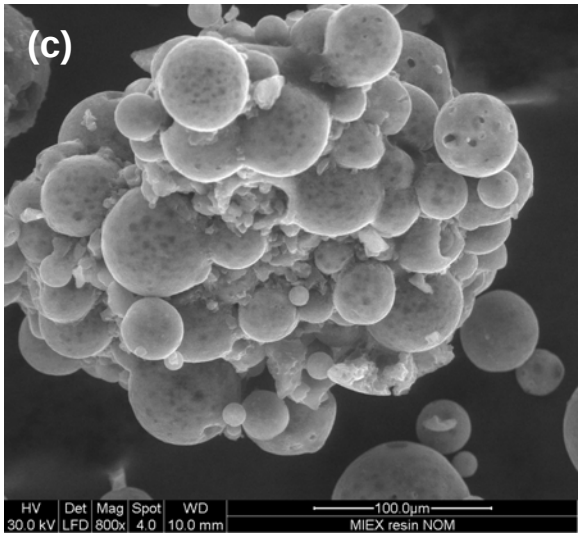
**Figure 8-5: SEM micrographs of C-18 resin after exposure to natural water and atrazine in MilliQ water.**



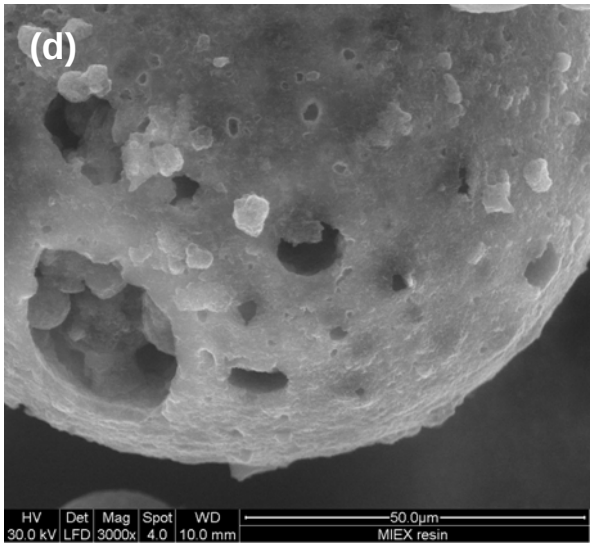

(a) MIEX© resin after exposure to MilliQ water




(b) MIEX© resin after exposure to atrazine solution (100 µg $L^{-1}$ in MilliQ water)




(c) MIEX© resin after exposure to natural water (DOM *ca.* 10 mg $L^{-1}$)




(d) MIEX© resin surface structure

**Figure 8-6: SEM micrographs of MIEX© resin after exposure to MilliQ water, 100 µg L-1 atrazine in MilliQ water and natural water (DOM *ca.* 10 mg $L^{-1}$).**

As illustrated in Figures 8-4 and 8-5, the Nexus© and C-18 resins had visible signs of atrazine sorption to the surface of the inspected beads when compared to the resins exposed to MilliQ water. However, as expected, the DOM was adsorbed on the MIEX© resin (Figure 8-6), but it was also observed to remove atrazine from solution (Figure 8-6(b)). In addition, the C-18 resin was observed to retain DOM after exposure to the natural water samples (Figure 8-5(c)). The NEXUS resin only retained atrazine (Figure 8-4).

As illustrated in Figure 8-3, when using the Nexus© resin in-line, the FICA system was re-configured from the reverse phase FIA system described in previous chapters, so that the

reagent was continuously pumped. Due to the continuous flow of the tris(2,2'-bipyridyl)ruthenium(III) reagent, there is a baseline chemiluminescence signal being generated from the reaction of tris(2,2'-bipyridyl)ruthenium(III) with the hydroxide in the buffer. This is evident in the MilliQ water trace displayed in Figure 8-7 where there is a significant drop in signal due to the dilution of the reagent upon mixing with the blank. However, in the presence of atrazine, a chemiluminescence response is clearly observed.

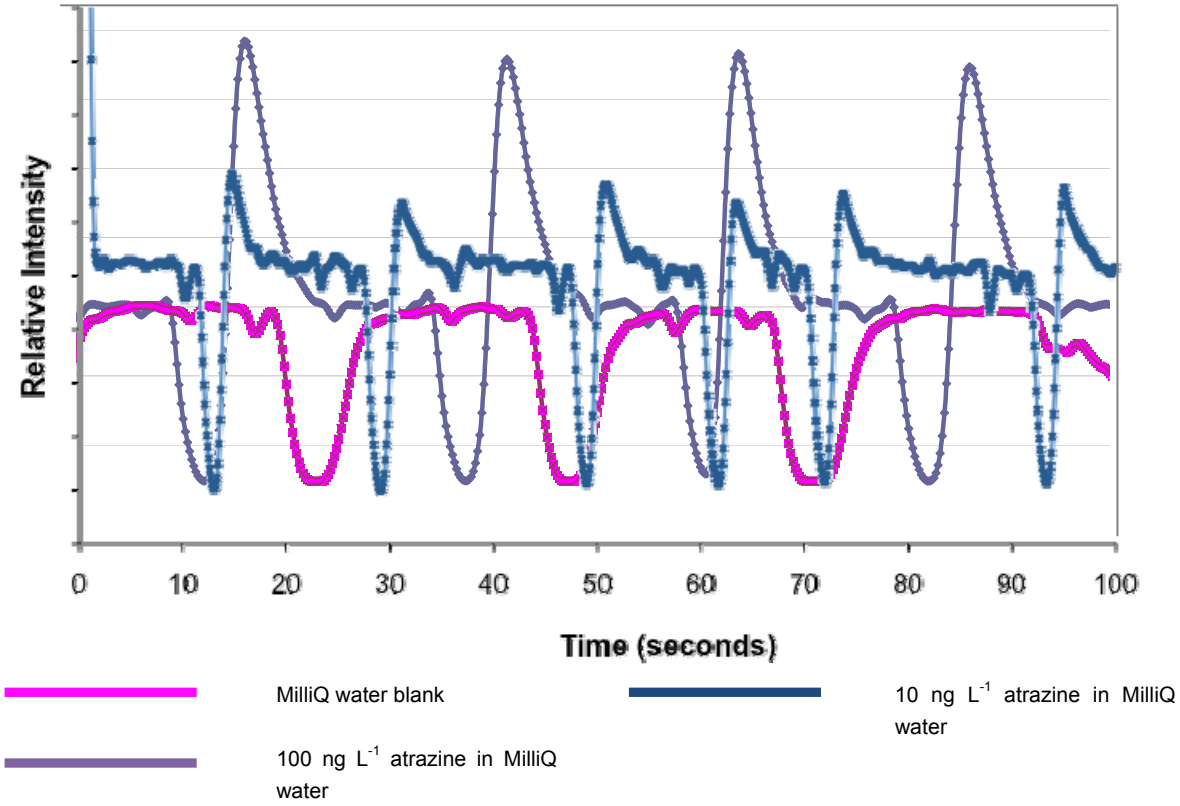



**Figure 8-7: FICA trace with in-line SPE using NEXUS© resin for the determination of atrazine in MilliQ water.**

A comparison of the described method with conventional flow injection analysis, direct injection HPLC, GC-NPD and GC-MS SIM indicates that in-line SPE flow injection eliminates interferences and lowers detection limits that are comparable to GC methods. While the in-line SPE flow injection method saves time, reduces sample volume and consumption of organic solvents (as illustrated in Table 8-3), it is important to note that the in-line SPE FIA system may respond to similar compounds and so all positive samples should be confirmed using a standard method (*i.e.,* GC-MS).

**Table 8-3: Comparison of analytical methods for pesticide determination in natural water.**

| Analytical Method | Vol. of Sample Required (mL) | Interferences Observed | MDL Range | Vol. organic solvent (mL) per sample | Loading/ preparation time (min) | Analysis time min/sample |
|---|---|---|---|---|---|---|
| Flow injection | 100 | NOM | µg/L | 0 | - | 1 |
| Flow Injection SPE | 100 | None* | ng/L | 0.1[a] | 20 | 1 |
| Direct injection HPLC | 0.5 | NOM | µg/L | 1.5[b] | - | 3 |
| GC-NPD EPA method 507 | 1000 | None* | µg/L | 90[c] | 30 | 57 |
| GC-MS SIM | 1000 | None* | ng/L | 60[d] | 30 | 60 |

***Note:*** *[a] 0.1 mL organic solvent accounts for the organic solvent used to elute the analyte from the SPE resin (C-18 and NEXUS© only); [b]4.5 mL organic solvent accounts for the organic solvent content of the mobile phase for the HPLC direct injection method based on the analysis time of 3 min; [c] 90 mL organic solvent accounts for the liquid – liquid extraction prescribed in the US EPA method 507. 1 L of filtered (0.45 µm) sample is extracted with three 30 mL aliquots of dichloromethane. The extract is reduced to a final volume of 1 mL prior to analysis by GC-NPD (NEMI, 2008; EPA, 2008); [d] 60 mL organic solvent accounts for 10 mL organic solvent used for the conditioning of the solid phase material and 50 mL used for elution of analytes from sorbent material. The extract was reduced to 100 µL prior to analysis by GC-MS selective ion monitoring (NEMI, 2008; EPA, 2008); and [*] no significant interference was observed from DOM. See table 3.*

The main objective of the SPE resins is to eliminate any interfering species from the analysis. A selection of cations, anions, DOM fractions and selected nitrogen containing molecules were analysed to ascertain maximum allowable limits (MAL) without any significant interference to analysis of the selected analyte; the methodology undertaken for the in-line SPE interference experiments was the same as the FICA interference study previously described in Chapter Seven. The difference between this study and the previous study is that this study quantifies the effect of potential interfering species with the extraction step used within the described systems. As shown in Table 8-4, there were no significant chemiluminescence interferences caused utilizing the Nexus© resins for the species tested.

**Table 8-4: Interference species study for the analysis of atrazine, hexazinone, and simazine by tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence with and without MIEX©, C-18 or Nexus© resin SPE.**

| Species | No SPE | NEXUS | ADWG[b] |
|---|---|---|---|
| | Concentration (Maximum allowable limit (MAL) (mg $L^{-1}$)) | | mg/L |
| $Ni^{2+}$ | **100** (100) | **100** (100) | - |
| $Ca^{2+}$ | **100** (100) | **100** (100) | 200 |
| $Zn^{2+}$ | 80 (100) | **100** (100) | 3 |
| $Mg^{2+}$ | 60 (100) | **100** (100) | 0.1 |
| $Cu^{2+}$ | 80 (100) | **100** (100) | 1 |
| $Cl^{-}$ | **100** (100) | **100** (100) | 250 |
| $K^{+}$ | **100** (100) | **100** (100) | - |
| $CO_3^{2-}$ | **100** (100) | **100** (100) | 200 |
| $HCO_3^{-}$ | **100** (100) | **100** (100) | 200 |
| $Na^{2+}$ | 80 (100) | **100** (100) | 180 |
| $Al^{3+}$ | **100** (100) | **100** (100) | 0.2 |
| $Fe^{2+}$ | 40 (100) | **100** (100) | 0.3 |
| $Fe^{3+}$ | 2 (100) | **100** (100) | 0.3 |
| Fulvic Acid | 2 (10) | **10** (10) | - |
| Humic Acid | 5 (10) | **10** (10) | - |
| Humate | 5 (10) | **10** (10) | - |
| Tryptophan | **25** (25) | 0 (25) | - |
| Tyrosine | 5 (25) | 0 (25) | - |
| Nitrate | **100** (100) | **100** (100) | 50 |
| Nitrite | **100** (100) | **100** (100) | 3 |
| Ammonia | 0.2 | 0.01 | 0.5 |
| pH | 5-8 | 4-10 | 6.5-8.5 |

***Note:*** *[b]Australian Drinking Water Guidelines (2004) minimum recommended guideline value for physical and chemical characteristics.* ***Bold*** *text indicates the maximum concentration tested that did cause a positive/negative interference.*

### 8.2.3. NEXUS© Resin Capacity

An atrazine adsorption – desorption study was carried out using a series of atrazine solutions (see Figure 8-8). For this batch study (n=5), 31 ± 8 mg of Nexus© was weighed and added to 100 mL of atrazine in MilliQ water (initial concentration confirmed by GC-MS; referred to as 'Initial Concentration' in Figure 8-8). The atrazine-Nexus solutions were then gently stirred on a flat bed agitator for 30 minutes prior to filtering. The resin was filtered and the filtrate extracted for atrazine and analysed ('Excess at Equilibrium'). The filtered resin was washed (100 mL MilliQ water) back into a beaker to assess the desorption of atrazine from the resin in clean MilliQ water ('Final concentration'). The resin was again filtered prior to an extraction being carried to assess the amount of atrazine remaining on the resin ('Extraction') which was calculated by difference. The high concentration at equilibrium suggests that the resin was saturated as intended.

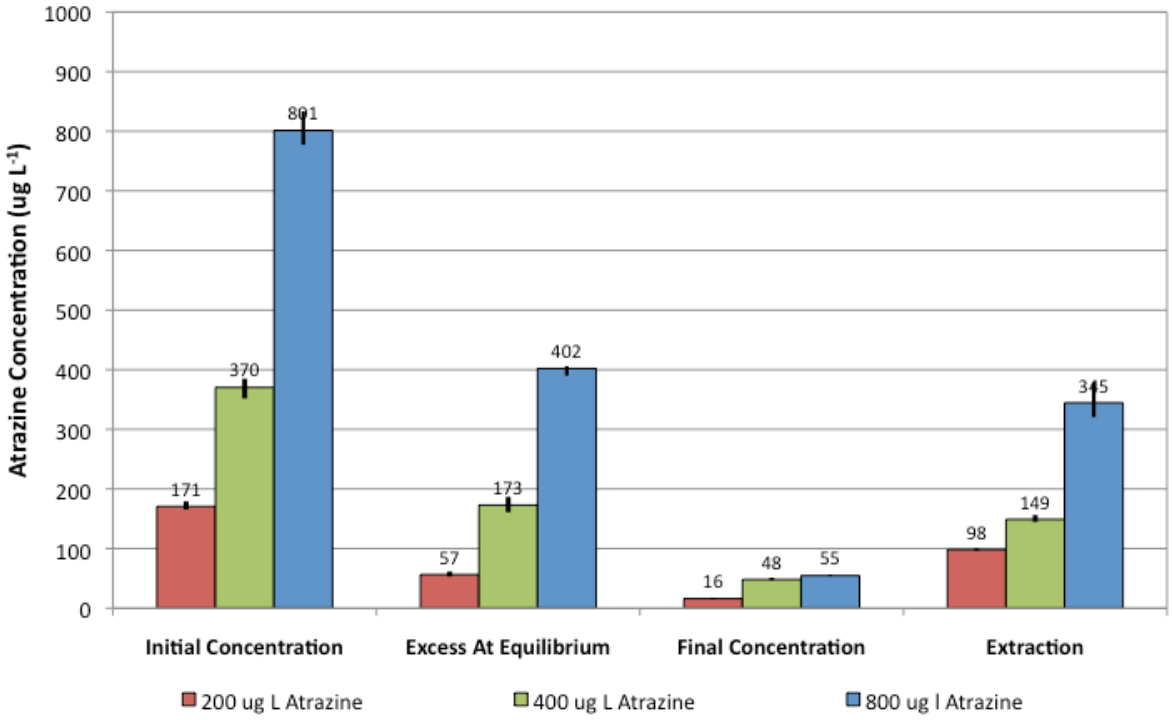



**Figure 8-8: Investigation of NEXUS absorption-desorption for atrazine in MilliQ water (n=5)**

In order to determine the capacity of the resin in the SPE cartridge, the capacity and the mass of the resin contained within the SPE cartridge was determined. The atrazine adsorption – desorption study was further extended in order to ensure saturation was achieved. As such, a series of atrazine solutions up to 12.5 mg $L^{-1}$ were analysed following the procedure described above.

Figure 8-9 presents the results from the extended adsorption-desorption study; the plot illustrates the multi layer adsorption characteristic of atrazine onto the Nexus© resin, which suggests the Nexus© resin is a high capacity resin – ideal for FIA with in-line SPE. From Figure 8-9, the amount of atrazine adsorbed by one gram of Nexus© resin can be determined by measuring the point at saturation.

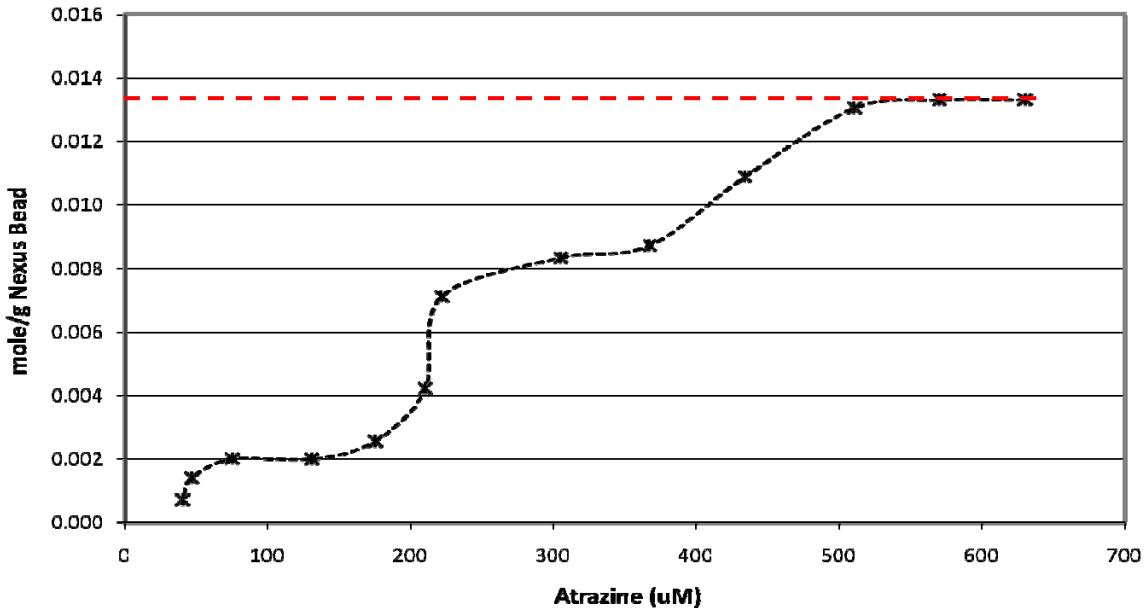



**Figure 8-9: Atrazine absorption isotherm using NEXUS resin (n=5).**

The mass of the resin was weighed by difference. An empty SPE cartridge was weighed and subtracted from the weight of a SPE cartridge filled with Nexus© resin (n=5). The mass of resin in the cartridge was then calculated to be 21 ± 4 mg.

Based on sorption properties established (see Figure 8-9), 0.0133 mole atrazine per g of Nexus© resin; the capacity of atrazine in the in-line SPE cartridge (*i.e.,* on *ca.* 23 mg Nexus) is calculated to be *ca.* 65 mg of atrazine (2.85 g of atrazine/g of resin).

## 8.3. Application

Six natural water samples (refer to Table 5-4) were analysed by in-line SPE flow injection chemiluminescence utilising the Nexus© resin for the determination of atrazine, hexazinone and simazine and validated using direct injection HPLC. Utilising the method described above, 4 samples per hour can be analysed.

The analysis of the six natural waters indicated that all but one water source was free from pesticide contamination. Hexazinone was detected at 0.12 ± 0.04 µg $L^{-1}$ in Sample 4 (see Figure 8-10). The natural water samples were individually spiked with 0.1 µg $L^{-1}$ atrazine, hexazinone and simazine, and then analysed. However, the spiked concentration was either at or below the detection limits of the direct injection HPLC method and so an SPE extraction was performed prior to analysis. The recoveries of all spiked samples ranged between 90-140% for the samples analysed by FICA (and 80-113% by HPLC; see Table 8-6). The larger recovery variation for the samples analysed by FICA is most likely due to the multiple SPE

cartridges used during the analysis (n=84 samples analysed; in total 14 SPE cartridges were used during the analysis). It was recommended by Varian (personal communication; Simpson, 2006) that the Nexus© resin should be refreshed after 10 extractions to ensure maximum extraction efficiency from the resin. In addition, the SPE manifold used were considered disposable (personal communication; Global FIA, 2007) but could be reused a limited number of times with new replacement frits. As such, and as a precautionary measure, the SPE cartridges were replaced every 6 extractions.

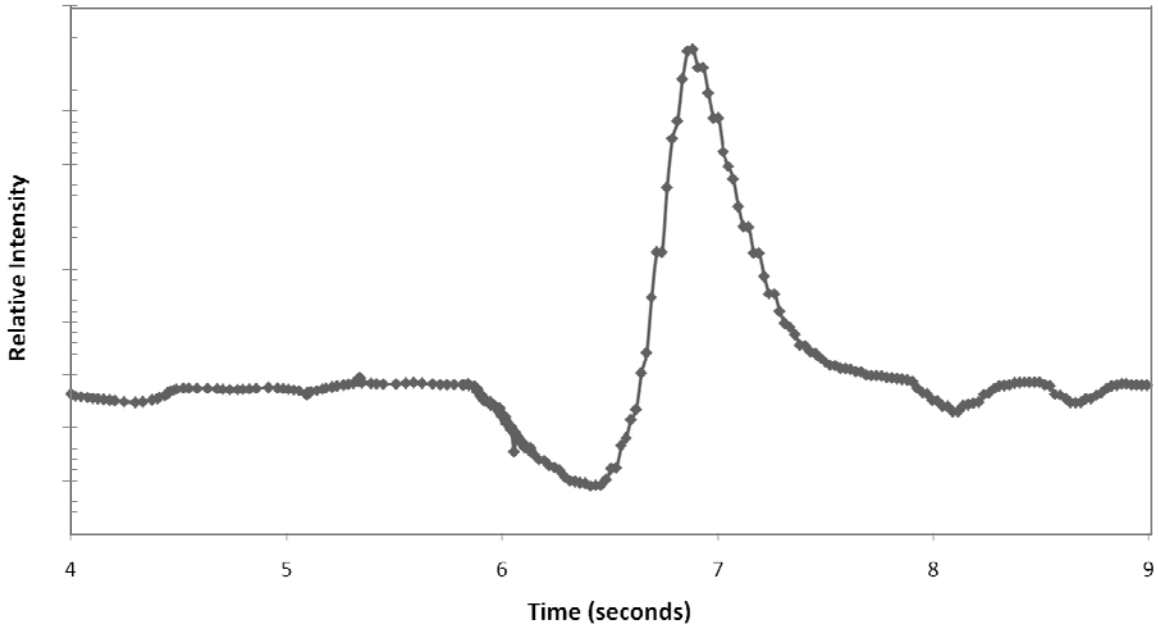



**Figure 8-10: Hexazinone determination in natural water (Sample 4, not spiked) by FICA with in-line SPE**

***Note:*** *FICA trace digitised from a paper by "GraphClick" (Arizona Software, Switzerland).*

**Table 8-5: Comparative analysis of natural water samples spiked with atrazine, simazine and hexazinone (recovery and statistical comparison of FICA and direct injection HPLC).**

| Sample | Blank | Natural samples Spiked with 0.1 µg $L^{-1}$ (n=3) | | |
|---|---|---|---|---|
| | | [Atrazine] | [Simazine] | [Hexazinone] |
| | **FIA** | **FIA (Rec.)** | **FIA (Rec.)** | **FIA (Rec.)** |
| | **HPLC** | HPLC (Rec.) | HPLC (Rec.) | HPLC (Rec.) |
| MilliQ | - | **0.10 ± 0.02 (100)** | **0.09 ± 0.02 (93)** | **0.11 ± 0.01 (100)** |
| | | 0.11 ± 0.02 (100) | 0.08 ± 0.01 (80) | 0.11 ± 0.01 (100) |
| 1 | **-** | **0.13 ± 0.03 (130)** | **0.14 ± 0.01 (140)** | **0.10 ± 0.03 (100)** |
| | | 0.09 ± 0.03 (87) | 0.11 ± 0.01 (110) | 0.10 ± 0.02 (97) |
| 2 | **-** | **0.09 ± 0.02 (90)** | **0.13 ± 0.02 (130)** | **0.09 ± 0.03 (90)** |
| | | 0.09 ± 0.02 (90) | 0.10 ± 0.03 (97) | 0.08 ± 0.02 (83) |
| 3 | **-** | **0.10 ± 0.03 (100)** | **0.11 ± 0.03 (110)** | **0.11 ± 0.03 (113)** |
| | | 0.10 ± 0.03 (103) | 0.11 ± 0.02 (107) | 0.10 ± 0.02 (103 |
| 4 | **0.12 ± 0.04** | **0.21 ± 0.03 (213)** | **0.20 ± 0.01 (200)** | **0.19 ± 0.01 (187)** |
| | 0.11 ± 0.01 | 0.19 ± 0.01 (193) | 0.20 ± 0.02 (200) | 0.19 ± 0.02 (187) |
| 5 | **-** | **0.10 ± 0.02 (100)** | **0.10 ± 0.02 (103)** | **0.09 ± 0.04 (93)** |
| | | 0.10 ± 0.02 (100) | 0.10 ± 0.02 (103) | 0.10 ± 0.02 (97) |
| **6** | **-** | **0.13 ± 0.03 (127)** | **0.12 ± 0.03 (117)** | **0.12 ± 0.04 (120)** |
| | | 0.10 ± 0.01 (100) | 0.11 ± 0.01 (113) | 0.10 ± 0.024 (98) |

## 8.4. Conclusion

The rapid analysis of pesticides in natural waters has previously proven to be difficult due to interfering species (*e.g.* DOM) masking any signal generated from the target analyte. However, with the incorporation of an in-line SPE flow injection extraction column, the analysis of atrazine, hexazinone, and simazine was successfully analysed with typical method detection limits as low as >0.01 µg $L^{-1}$ with no positive interferences observed Although the in-line SPE cartridge has capacity for larger extractions, due to the multi-layer adsorption capacity of the Nexus© resin, it enables the rapid analysis of small volumes (*i.e.,* 100 mL) to be analysed without an off-line extraction step (which can be time consuming and consumes larger volumes of solvent).

In the following chapter, a further modification to the system is discussed which incorporates a monolithic column (previously used in the modified direct injection HPLC, see Chapter Five) for the simultaneous determination and separation of atrazine (and its

metabolites), simazine and hexazinone in natural waters utilising an in-line SPE extraction manifold filled with Nexus© resin.

# CHAPTER NINE: FICA WITH IN-LINE SPE AND ANALYTE SEPARATION

The addition of an in-line extraction procedure (previously described in Chapter Eight) has significantly reduced the sample preparation time and detection limits obtained for atrazine, simazine and hexazinone in natural waters. However, one short coming of this FICA method is that it is, at best, a semi-qualitative (due to the selectivity of the chemiluminescent reagent, *i.e.,* aliphatic amines), quantitative analysis for the presence of a pesticide residues, with confirmation required using a methodology with separation capacity (*i.e.,* GC or HPLC). To overcome this shortcoming, which is common amongst FIA methods, low pressure separation columns are incorporated.

Traditionally, FIA systems rely on the selectivity of the reagents used and the mode of detection applied (after Adcock *et al.*, 2007). The development of conventional FIA systems and derivatives (e.g. sequential injection analysis and bead injection analysis) have been proposed for addressing new multi-analyte analytical challenges (Barnett *et al.*, 1999; Ruedas Rama *et al.*, 2004; Saurina, 2008; Spas & Ian, 2008). More recently, the hyphenation of instrumentation and the development of monolithic columns has enhanced the capability of flow systems, and opened up the prospect of FIA systems with in line separation (Solich *et al.*, 2008).

A caveat of such approaches requires separation columns of low hydrodynamic resistance to be selected, which makes columns like the monolithic column described in Chapter Five ideal and conventional packed columns undesirable. This chapter investigates the integration and application of a monolithic column into the FICA system previously described. As the suitability of the tris(2,2'-bipyridyl)ruthenium(III) reagent on the target analytes is not under question, neither is the handling of the sample matrix, extraction procedure, or effect of interfering species (as they have previously been addressed in the preceding chapters), this chapter focuses on the challenges of incorporating a monolithic column into a FICA system. In particular, what effect the mobile phase has on the chemiluminescence response and the reliability of the system.

## 9.1. Experimental

### 9.1.1. Solution Preparation

All stock solutions, chemicals and reagents were prepared as described in Chapter Three, unless otherwise stated. All natural water samples were filtered through a 0.45 µm nylon filter paper under vacuum (Millipore, Australia) prior to analysis.

### 9.1.2. FICA with In-Line SPE and Monolithic Separation

A 100 mL of sample (0.45 µm filtered) was propelled using two pumps in parallel (MilliGat, Global FIA, USA) through bridged PVC/PFTE tubing (1.85/0.76 mm i.d; Global FIA, USA) at 2.0 mL $min^{-1}$ through an extraction column; all other tubing was PFTE (0.76 mm i.d.). The extraction column (as illustrated in Figure 8.1) was packed with 21 ± 4 mg Nexus© resin for analyte isolation across a six-port injection valve (Rheodyne, USA); once the sample had passed through the column, the valve was switched – enabling the carrier mobile phase (acetonitrile-MilliQ water, 30:70% (v/v)) to elute the concentrate prior to separation on a monolithic column (RP-18e, 50-4.6 mm; Chromolith, Merck). The carrier stream then merges with the buffer stream and is dispersed by a mixing coil. The buffered sample then mixes with the chemiluminescence reagent which upon mixing produces light detected by the PMT (as previously described; see Figure 9-1 for a schematic of the modified FICA system designed for analyte pre-concentration).

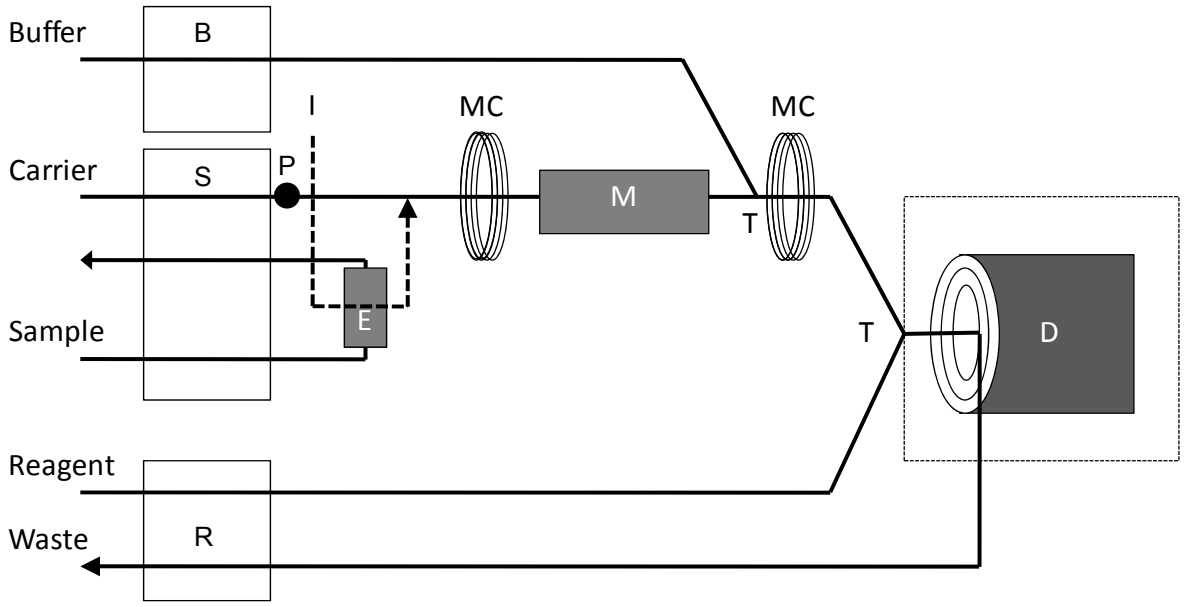



**Figure 9-1: FICA with in line SPE for the pre concentration and determination of pesticide residue in natural waters.**

***Note:*** *(R) Reagent stream pump (2 mM tris(2,2'-bipyridyl)ruthenium(III) in 20 mM $H_2SO_4$) pump; (S) Sample stream pump; (B) Buffer stream pump; (I) Injection valve (with extraction column); (P) pressure release valve, 100 psi (E) Nexus extraction column; (M) Monolithic column; (MC) Mixing coil; (T) T-piece; and (D) PMT.*

### *Impact of organic solvent on the chemiluminescence response*

While the application of tris(2,2'-bipyridyl)ruthenium(III) in FICA systems with separation capacity has been limited (e.g. Ridlen *et al.*, 1997; Adcock *et al.*, 2007), the application in HPLC and SIA systems is more common (Lee & Nieman, 1996; Satínský *et al.*, 2003; Satínský *et al.*, 2005; Satínský *et al.*, 2006 . Table 9-1 illustrates a selection of FIA, HPLC and hyphenated methods using tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence with varying mobile phase compositions; *Note, SIA methods are excluded, only continuous flow systems are considered.*

**Table 9-1: FIA, HPLC and Hyphenated FIA/HPLC systems that use tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence with in-line separation.**

| Analyte | Organic Solvent | Mobile phase | System | Reference |
|---|---|---|---|---|
| Aminopolycarboxylic acids | Water | 20 mM sulfuric acid (1 ml min−1). | HPLC | Pérez-Ruiz et al., 2007b |
| Amiodarone and Desethylamiodarone | Methanol | Methanol and 0.017 mol $L^{-1}$ ammonium sulfate buffer of pH 6.8. (92:8, v/v) | HPLC | Pérez-Ruiz et al., 2008 |
| Antihistamines | Acetonitrile | Acetonitrile-water (47:53, v:v) at pH 9.0; (35:65, v/v) at pH 7.0. | HPLC with post-column FIA detection | Holeman & Danielson, 1994 |
| Catechin | Acetonitrile | 20 mM phosphoric acid–acetonitrile (70:30, v/v). | HPLC | Kodamatani *et al.*, 2006 |
| Glyphosate and N-Phosphonomethyl Glycine | | HNOs mobile phase at pH 2.5 | Hybrid FIA/HPLC | Ridlen et al., 1997 |
| Nitrosamines | Acetonitrile | 5 mM acetate-acetonitrile (95:5, v/v). | HPLC with post-column FIA detection | Pérez-Ruiz *et al.*, 2005 |
| N-methylcarbamate | Acetonitrile | acetonitrile–water (30:70, v/v) | HPLC | Pérez-Ruiz et al., 2007a |
| Opiate alkaloids | Acetonitrile | 7–25% acetonitrile (line 1) in an aqueous solution adjusted to pH 2 with trifluoroacetic acid | Hybrid FIA/HPLC | Adcock et al., 2007 |
| Proline and Oxalate | Acetonitrile, methanol, acetone and 2-propanol | 0.1 M phosphate- with different percentages of organic solvents, (0 to 30%, v/v). | HPLC | Lee & Nieman, 1996 |
| Quetiapine | Methanol | Methanol (gradient elution 15-100%) -trifluoroacetic acid (0.1%, v/v) | HPLC | Bellomarino *et al.*, 2009 |
| Triethylamine, Tri-n-propylamine, and Tri-n-butylamine | Acetonitrile | 40 mM phosphate – acetonitrile (99:1, v/v). | HPLC | Yamazaki et al., 1998 |
| Tryptophan and Tyrosine | Acetonitrile | water–acetonitrile (83:17) | HPLC | Bolden & Danielson, 1998 |

As illustrated in Table 9-1, a variety of organic mobile phases were used for the successful determination of a range of analytes using tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence within aqueous solutions. Interestingly, Lee & Nieman (1996) reported that the use of organic solvent increased the chemiluminescence intensity of tris(2,2'-bipyridyl)ruthenium(III) for proline and oxalate determination (the signal was increase by a factor of three when acetonitrile was used); similar results were obtained for methanol, acetone, and 2-propenol. As the mobile phase of acetonitrile-water (30:70, v/v) was used successfully in Chapter Five for the separation of atrazine, simazine and hexazinone using

the same monolithic column, it was decided that the same methodology would be applied to a FICA system.

### *Impact of multiple pumps*

As stated previously, FICA systems with in-line separation requires the use of low pressure columns, while the benefits of monolithic columns were discussed in Chapter Five (*e.g.* low pressure, high capacity and high throughput). The impact of pressure is still an important characteristic that needs to be minimised (Adcock *et al.*, 2007; Solich *et al.*, 2008). As such, the pumps used to control the flow of the sample and carrier streams were two MilliGat pumps, positioned in parallel. These pumps are rated to withstand a back pressure of 100 psi. As a preventative measure, a pressure release valve was positioned between the sample extraction injection valve and the MilliGat pumps to ensure the back pressure was not excessive. In addition, the waste stream was forward propelled (*i.e.,* pumped out of the system). All subsequent pumps were peristaltic pumps. The use of multiple pumps, pumps in parallel and slightly larger tubing (0.76 mm i.d.) ensures the pressure on each pump is reduced while maintaining the required flow rate needed for tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence.

The flow rate of the system was maintained at a combined flow rate of 8 mL $min^{-1}$, and as such each pump in parallel is set at a flow rate of 2 mL $min^{-1}$

### 9.1.3. Direct Injection HPLC (Sample Validation)

Samples were validated using a direct injection HPLC system described in Chapter Five.

## 9.2. Results and Discussion

### 9.2.1. Effect of Mobile Phase Composition

The effect of the mobile phase composition (acetonitrile-water (30:70, v/v)) on the chemiluminescence signal was observed for atrazine, hexazinone and simazine. The effect of the mobile phase was similar to the effect observed when analysing the SPE extracts described in Chapter Seven and the in-line SPE system described in Chapter Eight; the intensity was enhanced by ca. 23%.

### 9.2.2. Detection of Multiple Pesticides

A series of 100 mL atrazine, simazine and hexazinone solutions ranging in concentration from 1 ng $L^{-1}$ to 10 000 ng $L^{-1}$ in MilliQ water were prepared. The samples were analysed

using the system described in Figure 10-1, with thorough washing of the resins conducted between each analysis. For illustrative purposes, Figure 9-3 presents an example trace utilizing the FICA system with the monolithic column and the comparative HPLC system described in Chapter Five.

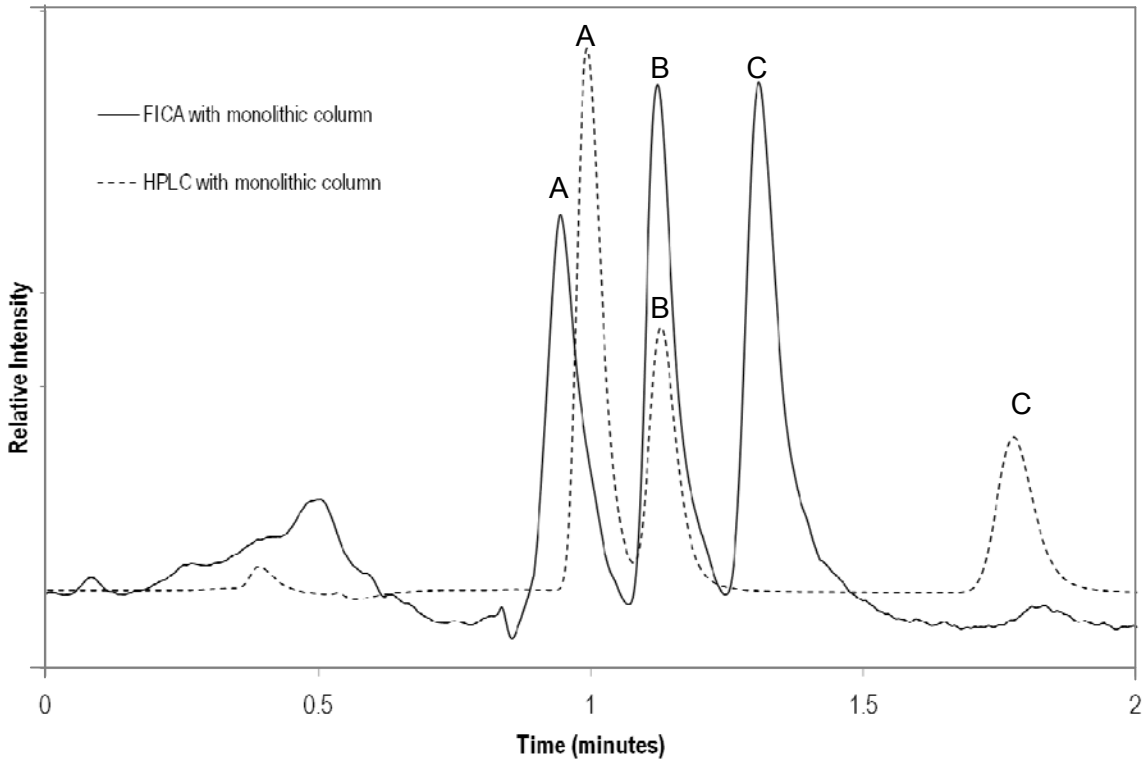



**Figure 9-2: FICA monolithic chromatogram of atrazine, simazine and hexazinone in MilliQ water**

***NOTE:*** *Chromatogram of 50 µg $L^{-1}$ standard solution of atrazine, simazine and hexazinone; peaks identified as (A) hexazinone; (B) simazine; (C) atrazine.*

Table 9-2 summarises the analytical figures of merit achieved for the determination of atrazine by FICA with in-line SPE and monolithic separation.

**Table 9-2: Method detection limits (MDL) for Nexus© in-line SPE and monolithic separation with tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence for 100 mL samples in MilliQ water.**

| Analyte | Method Detection Limit (MDL) ng/L | ADWG[a] ng/L |
|---|---|---|
| Atrazine | 27 ± 6 | 100 (40) |
| Hexazinone | 60 ± 14 | 2 000 (300) |
| Simazine | 39 ± 12 | 500 (20) |

***Note:*** *[a]Australian Drinking Water Guidelines (2004) recommended limit of detection (and health value based on 10% of allowable daily intake)*

### 9.2.3. Application to Natural Waters

The presence of organic matter has previously been found to mask the chemiluminescence signal generated by target pesticides using the FICA system described in previous chapters; this was overcome by incorporating SPE. The next challenge is to analyse multiple pesticide species in natural waters by utilising a monolithic column as demonstrated in neat samples (Figure 9-3). As such, six natural water samples (refer to Table 5-4) were analysed by in-line SPE flow injection chemiluminescence utilising a monolithic column for the determination of atrazine, hexazinone and simazine and validated using direct injection HPLC.

The analysis of the six natural waters confirmed the findings presented in Chapter Eight; hexazinone was identified as a contaminate in Sample 4. The natural water samples were individually spiked with 0.1 µg $L^{-1}$ atrazine, hexazinone, and simazine; and analysed; SPE extraction was performed prior to analysis by HPLC. The recoveries of all spiked samples ranged between 99-170% for the samples analysed by FICA (and 80-113% by HPLC; see Table 9-3). The larger recovery variation for the samples analysed by FICA is most likely due to the multiple SPE cartridges used during the analysis, as described previously.

**Table 9-3: Comparative analysis of natural water samples spiked with atrazine, simazine and hexazinone (recovery and statistical comparison of FICA with monolithic separation and direct injection HPLC).**

| Sample | Natural samples Spiked with 0.1 µg $L^{-1}$ (n=3) | | |
|---|---|---|---|
| | [Atrazine] | [Simazine] | [Hexazinone] |
| | **FICA (Rec.)**<br>HPLC (Rec.) | **FICA (Rec.)**<br>HPLC (Rec.) | **FICA (Rec.)**<br>HPLC (Rec.) |
| MilliQ | **0.11 ± 0.03 (110)**<br>0.11 ± 0.02 (100) | **0.10 ± 0.01 (100)**<br>0.08 ± 0.01 (80) | **0.11 ± 0.02 (104)**<br>0.11 ± 0.01 (100) |
| 1 | **0.14 ± 0.02 (140)**<br>0.09 ± 0.03 (87) | **0.15 ± 0.03 (150)**<br>0.11 ± 0.01 (110) | **0.11 ± 0.02 (109)**<br>0.10 ± 0.02 (97) |
| 2 | **0.13 ± 0.04 (130)**<br>0.09 ± 0.02 (90) | **0.16 ± 0.04 (160)**<br>0.10 ± 0.03 (97) | **0.11 ± 0.04 (110)**<br>0.08 ± 0.02 (83) |
| 3 | **0.13 ± 0.04 (130)**<br>0.10 ± 0.03 (103) | **0.14 ± 0.02 (140)**<br>0.11 ± 0.02 (107) | **0.14 ± 0.05 (140)**<br>0.10 ± 0.02 (103 |
| 4 | **0.12 ± 0.02 (120)**<br>0.19 ± 0.01 (193) | **0.13 ± 0.05 (130)**<br>0.20 ± 0.02 (200) | **0.25 ± 0.06 (250)**<br>0.19 ± 0.02 (187) |
| 5 | **0.11 ± 0.03 (110)**<br>0.10 ± 0.02 (100) | **0.11 ± 0.06 (105)**<br>0.10 ± 0.02 (103) | **0.10 ± 0.03 (99)**<br>0.10 ± 0.02 (97) |
| 6 | **0.17 ± 0.05 (170)**<br>0.10 ± 0.01 (100) | **0.16 ± 0.05 (160)**<br>0.11 ± 0.01 (113) | **0.16 ± 0.02 (160)**<br>0.10 ± 0.024 (98) |

### 9.2.4. Interferences

In addition to the interference study carried out in Chapter Eight, an investigation of '*like*' compounds, in terms of peak resolution and retention time, was undertaken utilising known

atrazine metabolites. Figure 9-4 illustrates the co-elution of peaks between atrazine, simazine, and hexazinone and the metabolites atrazine-2-hydroxy, atrazine-desisopropyl, and atrazine-desethyl.

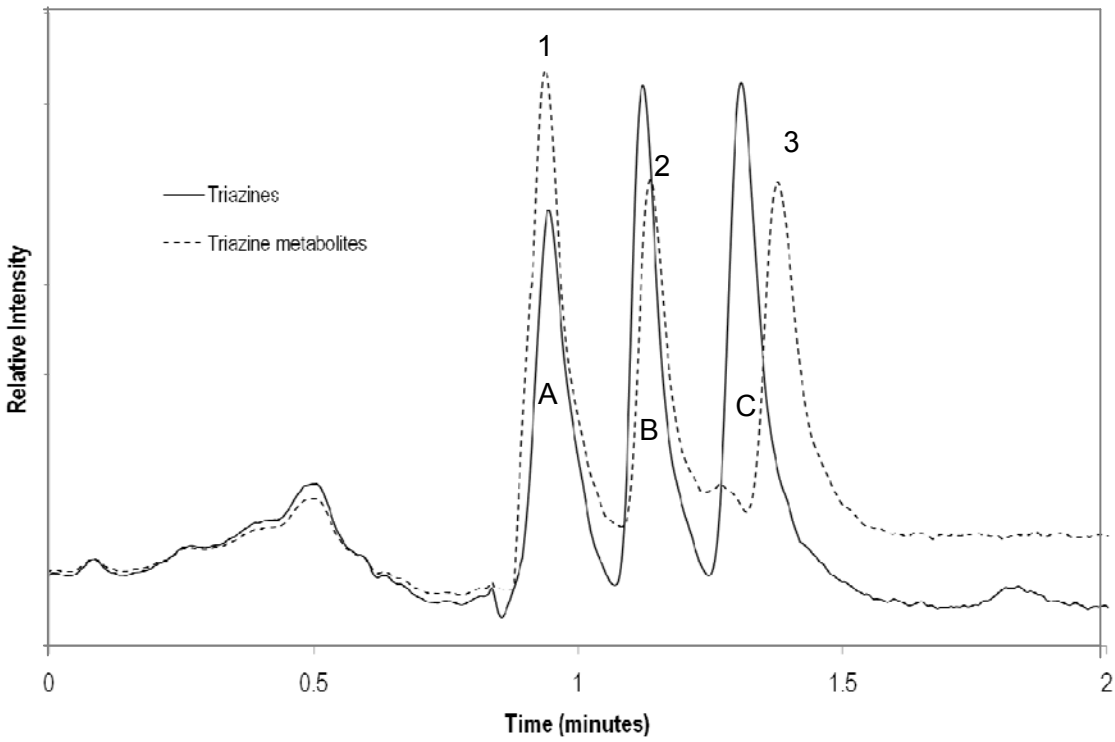



**Figure 9-3: Investigation of potential interference of triazine metabolites.**

***NOTE:*** *peaks identified as (A) hexazinone; (B) simazine; (C) atrazine; (1) atrazine-2-hydroxy; (2) atrazine-desisopropyl; (3) atrazine-desethyl. Mobile phase 30:70 (ACN:$H_2O$), flow rate 8.0 mL $min^{-1}$. 50 µg $L^{-1}$ pesticide stock solution.*

## 9.3. Conclusions

The benefits of a monolithic column (*e.g.* low pressure chromatographic separation) were merged with the advantages of the in-line SPE in order to create a hybrid FICA system analogous to a low pressure HPLC system. While the system has not been optimised following a formal optization procedure, the optimal conditions identified in previous experiments were transposed to the modified instrument that incorporated a monolithic column and enabled atrazine, simazine and hexazinone to be detected simultaneously with chromatographic differentiation. The method detection limits for atrazine, simazine and hexazinone were 27 ± 6, 39 ± 12 and 60 ± 14 ng $L^{-1}$, respectively.

The presence of the triazine metabolites atrazine-2-hydroxy, atrazine-desisopropyl, and atrazine-desethyl would cause interference if in solution; however, this could be overcome through formal optimisation of the method.

# CHAPTER TEN: INVESTIGATION OF INTERFERENCE USING 3D FLUORESCENCE

This chapter presents an investigation into the possible interferences of DOM in FICA using 3D excitation-emission fluorescence spectroscopy.

## 10.1. Experimental

All chemicals and reagents used throughout this chapter are presented in Chapter Three (sections 3.1.2 and 3.1.3).

### 10.1.1. Fluorescent Standards

Humic-like (humic acid, fulvic acid and tannic acid) and protein-like (tryptophan, and tyrosine) standards were prepared daily in MilliQ water (5.0 mg $L^{-1}$) and adjusted to pH 7 (using drop-wise addition HCl or NaOH, 0.1 M (AR)). All pesticides, cations and anions were prepared as described in Chapter Three, unless otherwise stated.

Prior to analysis the pH of the natural water samples were adjusted to 7 (using a drop-wise addition HCl or NaOH, 0.1 M (AR)) after being brought to room temperature (*ca*. 22˚C; variation in colloidal material, pH and temperature have been demonstrated to effect the reproducibility of the DOM fluorescence spectra; see Spark & Swift, 1994; Baker *et al.*, 2007; Hudson *et al.*, 2007). Natural water samples and standards were all filtered (hydrophilic polyvinylidene fluoride (PVDF) MF membranes (Durapore GVWP, 0.22 µm, Millipore)).

All natural water samples were stored in 200 mL glass amber bottles (pre cleaned with Pyroneg, Johnson Diversy, Australia, and triple rinsed with MilliQ water).

### 10.1.2. 3DEEM Fluorescence Spectroscopy

3DEEM fluorescence spectroscopy was undertaken with a fluorescence spectrophotometer (Perkin Elmer, Model LS 50B) operating FL WinLab (Perkin Elmer, USA) with quartz cells (10 mm x 10 mm x 70 mm). Wavelengths ranged from 200 to 600 nm for excitation (5 nm bandwidth), and from 200 to 600 nm for emission (5 nm bandwidth).

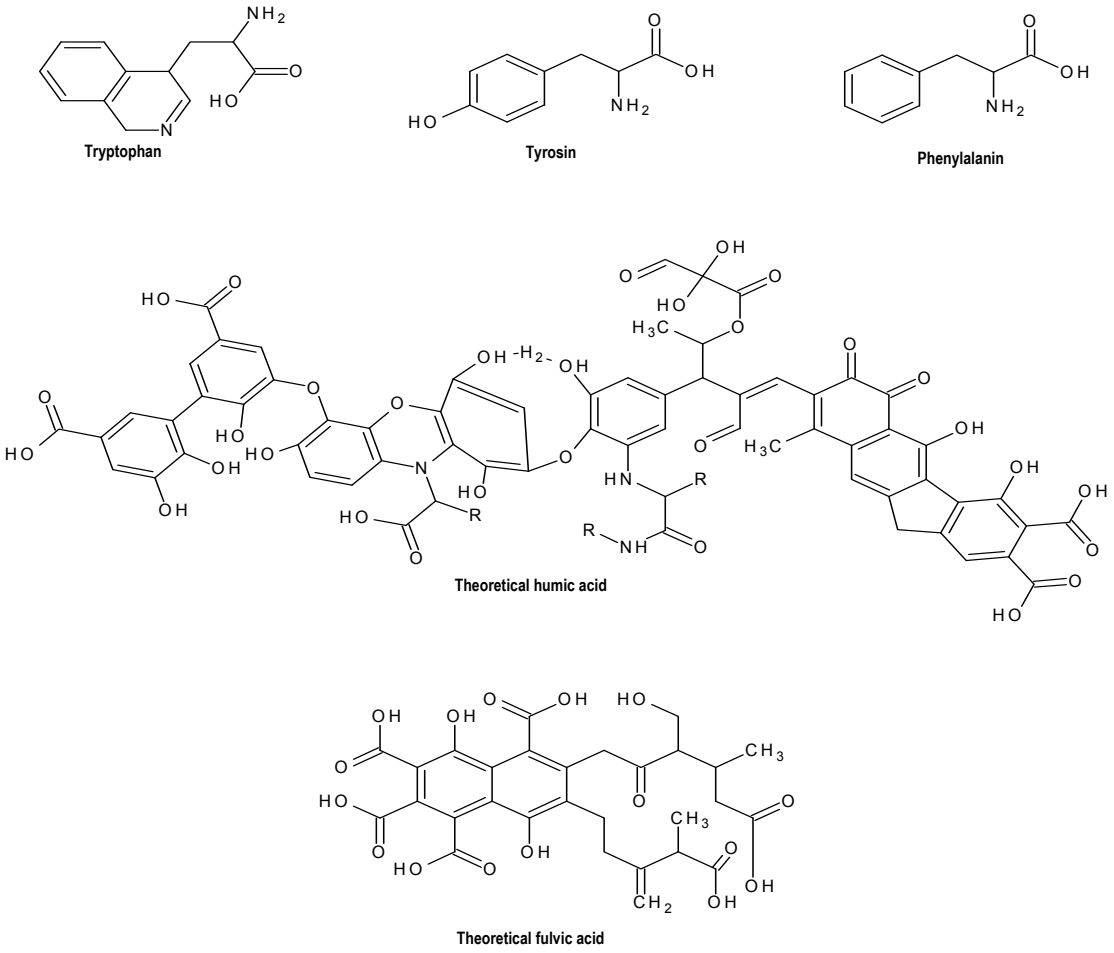
### *Fluorescence fingerprint analysis*

Organic matter fluorescence occurs when a loosely held electron in an atom or a molecule is excited to a higher energy level by the absorption of energy and fluoresces when that energy is lost as light when the electron returns to its original energy state (*i.e* stable ground state). The wavelength at which the absorption (excitation) and emission occurs is very specific to each molecule; molecules that absorb light are termed chromophores while molecules that re-emit light are fluorophores (after Chow *et al.*, 2006; Hudson *et al.*, 2007).

The most common fluorescence tool used today for the assessment of DOM is 3DEEM fluorescence spectroscopy, where both the emission and excitation wavelengths (along with fluorescence intensity) are scanned synchronously over a range of wavelengths. Unlike synchronous fluorescence scanning (*i.e.,* where an emission profile is monitored over a wavelength range for a fixed excitation wavelength or a stepped wavelength range; also known as SFS), 3DEEM fluorescence is able to scan and capture both the same excitation and emission wavelength at the one time. (SFS requires a minimum difference of 12-60 nm between the excitation and emission wavelength).

### *Common DOM fluorescence signatures*

The most common DOM fluorescent fractions include humic acid-like substances (humic and fulvic acid) and protein-like substances (in the form of proteins and peptides: tryptophan, tyrosine and phenylalanine); these fluorescent fractions are classed as aromatic compounds comprising a higher degree of energy sharing due to their un-paired electron structure in their associated carbon ring (after Hudson *et al.,* 2007; see Figure 10-1 for an illustration of these structures).




**Source:** Hudson *et al.,* 2007

**Figure 10-1: Common DOM fluorescent fractions in natural waters**

The presence and interpretation of protein-like fluorophores present within a DOM fluorescence spectrum has been widely debated; Yamashita & Tanoue(2003) state that it is a result of 'free' amino acids in the DOM pool, Determann *et al.* (1998) claim it is partially from amino acids bound in proteins or microbe cell walls, while Elliott *et al.*(2006) further state it is evidence of bacteria or from a bacterial origin.

Due to the difficulties in naming and identifying each fluorophore, these molecules are grouped into a series of common fluorophore classes, namely: humic acid-like and protein-like. Table 10-1 illustrates the known fluorophores in natural waters investigated in this study and the typical regions of the EEM spectra in which they occur (Figure 10-2).

**Table 10-1: Common fluorophore names and position in 3DEEM spectra**

| Fluorphore Type | Excitation/Emission (Ex/Em) wavelength | Reference |
|---|---|---|
| Humic-like (A) | 237-260/400-500 | Parlanti *et al.*, 2000; Henderson *et al.*, 2009 |
| Humic-like (C) | 300-370/400-500 | Parlanti *et al.*, 2000 |
| Protein-like (tyrosine-like; B1 and B2) | 225-237/309-321 & 275/310 | Parlanti *et al.*, 2000 |
| Protein-like (tryptophan –like; T1 and T2) | 225-237/340-381 & 275/340 | Parlanti *et al.*, 2000 |

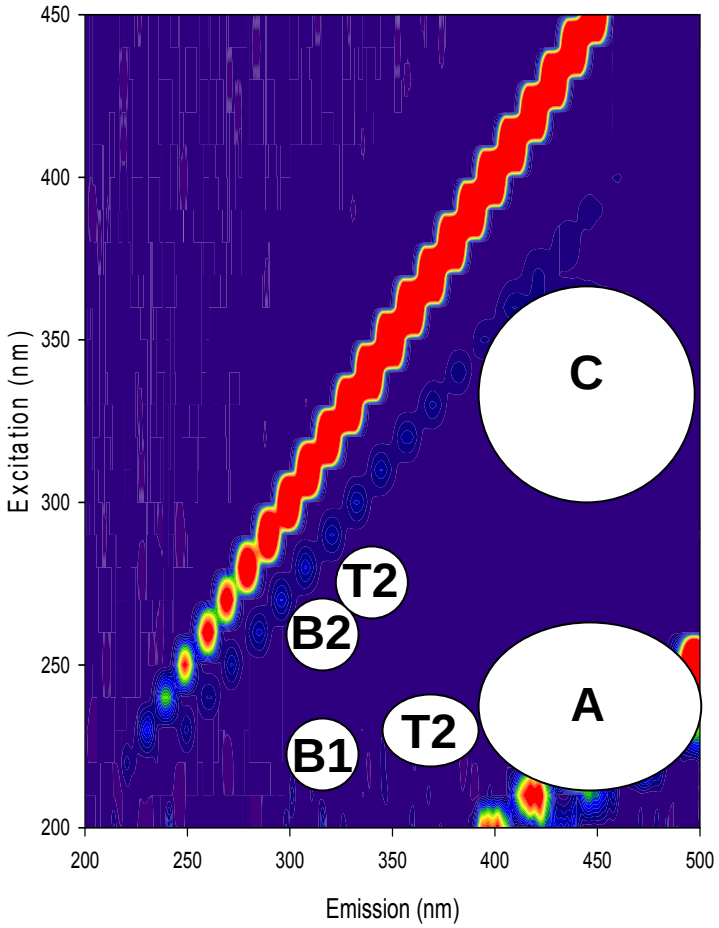



**Figure 10-2: EEM spectra illustrating common EEM fluorophores (after Hudson et *al.*, 2007)**

### *Interpreting 3DEEM signatures*

There are three common methods for interpreting EEM spectra: visual identification of fluorescence peaks (as conceptually illustrated in Figure 10-2); measuring the intensities of specific peaks (where the ratio of peaks is used for monitoring and discriminating between waters, see Baker & Spencer, 2004); and dividing EEMs into defined sections associated with specific fluorophores (each section is then integrated and normalised within these boundaries) (Henderson *et al.*, 2009).

The visual identification of peaks is qualitative, it does not demonstrate changes statistically or illustrate enough discrimination between samples over time. In contrast, techniques

where EEMs are divided, integrated and normalised are criticised for making minimal use available data. Characteristics of interest such as peak location, which may shift due to the presence of overlapping spectra or chemical interference, are not retained (Henderson *et al.*, 2009). As such, the data analysis approach undertaken in this study incorporates measuring peak intensities and noting shifts in EEM profiles (after Baker & Spencer, 2004).

### *Application of 3DEEM to natural waters*

Fluorescence fingerprinting has been utilised by numerous authors to monitor water quality both spatially and temporally by observing changes in specific fluorescence signatures (*e.g.* fluctuations in DOM, see Hudson, 2007). DOM in environmental waters originates from a variety of sources (Chow *et al.*, 2006) and the relative contribution from each source depends upon the location and environmental conditions both within and surrounding the water body. The application of fluorescence spectroscopy has been widely applied to a variety of aqueous media: marine, fresh and recycled waters, primarily for the characterisation of DOM (as described in more detail below). Fluorescent fingerprinting provides information on the source of the water, for the monitoring and understanding of DOM transformations, and for monitoring of contaminants (Hudson *et al.*, 2007; Henderson *et al.*, 2008).

As indicated previously, many researchers have utilised EEM fluorescence to characterise environmental waters; Jiang *et al.* (2008) characterised and identified the source of DOM in marine waters in the Bohai Sea (China) at various depths; the DOM profile was cross validated by comparing the movement of DOM within seawater with a computational model of ocean currents. Mostofa *et al.* (2007) characterised DOM in groundwater, lake and river water, and illustrated a significant variation across each water body type relating the differences in the EEM spectra as a result of each sample comprising varying anion concentrations; however, no correlation between anion concentration and fluorophore intensity was observed (Mostofa *et al.*, 2005; Jiang *et al.*, 2008). Some researchers have utilised 3DEEM fluorescence for the investigation of pollutants in waters, identifying distinct protein-like fluorophores (resulting from pathogens) and specific fluorescent signatures from landfill (*e.g.* fluorescence signature of naphthalene) (Baker & Curry, 2004; Jiang *et al.*, 2008). Other researchers have tried to obtain more detailed information from EEM spectra by analysing specific fractions of waters (fractions obtained by passing waters through a series of XAD and ion exchange resins), to better characterise the composition of DOM: Santin *et*

*al.* (2009) investigated humic and fulvic fractions from estuarine sediments in Spain in an attempt to map its origins; Baker & Spencer (2004) characterised natural waters in southern China containing mainly fulvic acid fractions (*ca.* 50%) with the remainder consisting a mixture of hydrophobic neutrals, hydrophilic acids, hydrophilic bases and humic acids. Chen *et al.* (2003) fractionated DOM into polyphenolic-rich and carbohydrate-rich sub fractions to assess the structural and functional properties of DOM confirming previous studies on the heterogeneity of DOM in natural waters (Santin *et al.*, 2009).

#### Effect of metals, cations and anions

Investigation into the effects of metal ions, and by extension the combination of metal ions and cations with DOM is rather limited; some researches have investigated the effect of known metals that are used in coagulation processes (*e.g.* iron and aluminium) utilising EEM to illustrate the enhancement or quenching of fluorophores, indicating the formation of more complex molecules (see Table 10-2 for a summary of selected studies).

**Table 10-2: Studies on the effects of metal ions of 3DEEM spectra**

| Ion | Water type | Effect on Excitation/Emission spectra | Reference |
|---|---|---|---|
| Copper (II) | Fresh water | Blue-shift in humic-like fluorophore (a) | Wu *et al.*, 2001; Wang *et al.*, 2009a |
| Iron oxide | Standard | Quenching of humic-like fluorophores | Manciulea *et al.*, 2009 |
| Aluminium | Fresh water | Enhancement and Quenching of humic-like fluorophores | Parlanti *et al.*, 2000 |
| Mercury (II) | Fresh water | Quenching of humic- and protein-like fluorophores | Fu *et al.*, 2007 |
| Calcium with Mercury (II) | Fresh water | Enhancement of humic- and protein-like fluorophores | Fu *et al.*, 2007 |
| Chloride with Mercury (II) | Fresh water | Enhancement of humic- and protein-like fluorophores | Fu *et al.*, 2007 |
| Iron oxide (II) | Standard/Fresh water | No observed effect | Pullin *et al.*, 2007 |
| Iron oxide (II) | Standard/Fresh water | Quenching of humic-like fluorophores | Pullin *et al.*, 2007 |
| Copper (II) | Fresh water | Quenching of humic- and protein-like fluorophores | Yamashita & Jaffe, 2008 |
| Mercury (II) | Fresh water | Quenching of humic- and protein-like fluorophores | Yamashita & Jaffe, 2008 |

#### Pesticide analysis

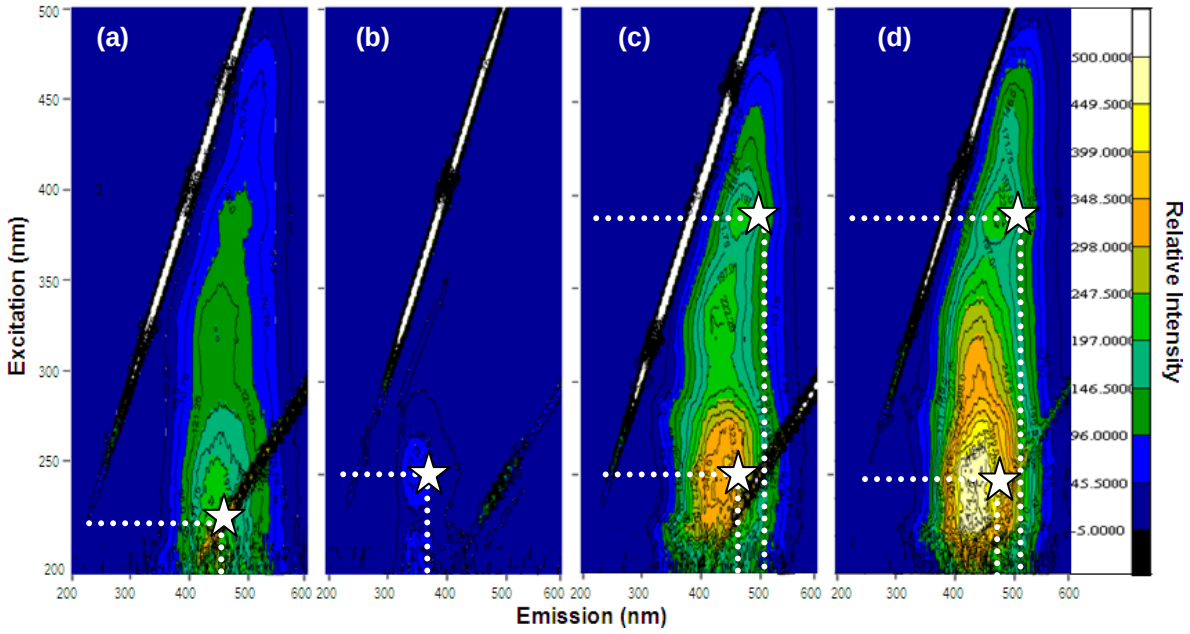
Spark & Swift (1994) investigated the interaction between pesticides (atrazine, 2,4-D, isoproturon and paraquat at 1 mg $L^{-1}$) with humic substances using fluorescence over the emission range of 400 to 600 nm (at an excitation wavelength of 340, 390 and 450 nm). It was observed that all pesticides studied were adsorbed to the humic material (solid and aqueous); however, the point of interaction was not determined (Spark & Swift, 1994).

Huang *et al.* (2008) characterised triazines in effluent from a pesticide manufacturing treatment plant using 3DEEM. Although they were able to identify triazines present in the treatment process effluent and use the technique to assess the percentage removal, it is important to note that concentrations were significantly higher than expected environmental levels (*e.g. ca.* 90 mg $L^{-1}$) and the effluent was free from naturally occurring DOM (Huang *et al.,* 2008).

While EEM has the potential to provide valuable information on natural waters in terms of DOM characteristics, it also has the potential to illustrate the interactions between DOM and pesticides in the environment taking into to account the effect of various cations, anions and metals commonly found in solution. This section presents the work completed on the investigation of the interaction of the selected pesticides (*i.e.* atrazine, simazine, hexazinone, monocrotophos and dicrotophos) in the sampled natural waters to determine the potential for the formation of pesticide-DOM complexes, and assess the application of 3DEEM fluorescence as a tool for pesticide screening via fluorescence fingerprinting.

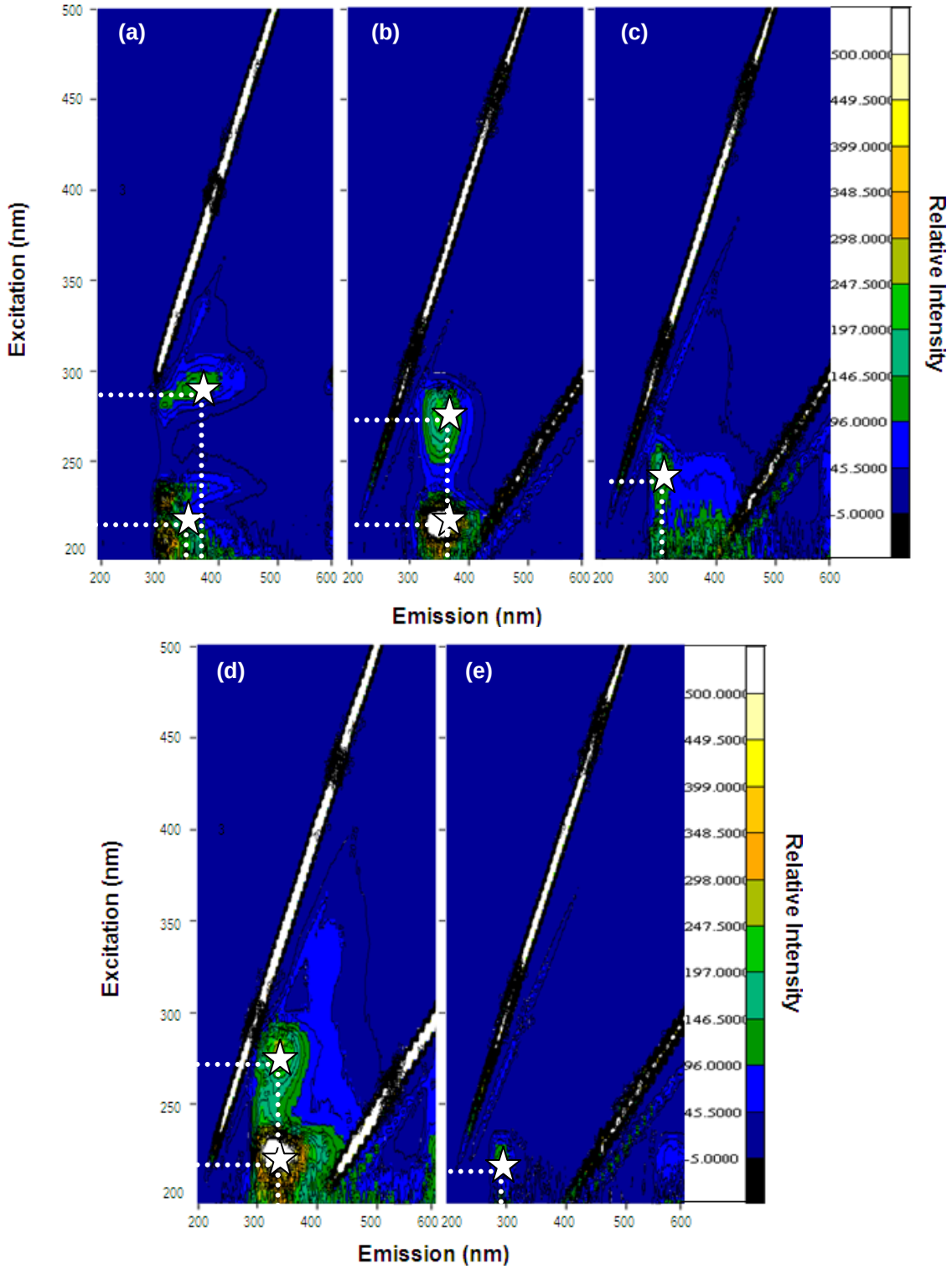
## 10.2. Results and Discussion

A series of humic-like and protein-like standards were prepared and compared to the fluorophore signatures found in the literature. Figure 10-3 illustrates the humic-like fluorophores from scientific grade humic acid and tannic acid along with commercially available fertiliser solutions of humate (humic acid) and fulvic acid (sourced from Omnia, Australia) in MilliQ water, while Figure 10-4 illustrates the fluorophores observed in analytical grade tryptophan and tyrosine in MilliQ water.




## Figure 10-3: Humic-like fluorophores

***NOTE:*** *(a) humic acid standard (Ex-225nm; Em-450nm), (b) tannic acid standard (Ex-250nm; Em-350nm), (c) humate (Ex-250nm; Em-425nm. Ex-380nm; Em-480nm), and (d) fulvic acid (Ex-250nm; Em-425nm. Ex-380nm; Em-480nm). All solutions were 5 mg $L^{-1}$ in MilliQ water. Stars indicate known fluorophores, refer to Table 10-1.*




## Figure 10-4: Protein-like fluorophores

***NOTE:*** *(a) Tyrosine standard (Ex-220nm; Em-340nm. Ex-280nm; Em-380nm), and (b) Tryptophan standard solutions (Ex-225nm; Em-350nm. Ex-275nm; Em-350nm); additional protein-like compounds were assessed, namely: (c) Bacteria commonly found within water distribution systems (Ex-280nm; Em-300nm)(sourced from Solraska et al., 2010, and (d) Microbes commonly found in drinking water catchments (Ex-225nm; Em-340nm. Ex-275nm; Em-340nm (sourced from Beale et al., 2010)), and (e) L-proline standard (Ex-225nm; Em-290nm). Valine, alanine, glycine amino acids were analysed but were not observed to fluoresce, as expected. All solutions were 5 mg $L^{-1}$ in MilliQ water. Stars indicate known fluorophores.*

As illustrated in Figures 10-3 & 10-4, the humic-like and protein-like fluorophores found in the literature were observed in the standard solutions analysed. Table 10-3 summarises the observed and known fluorophores in the literature for each solution.

**Table 10-3: Common fluorophore names and position in 3DEEM spectra**

| Standard | Fluorophore Type | Excitation/Emission (Ex/Em) wavelength^ | Literature Excitation/Emission (Ex/Em) wavelength* |
|---|---|---|---|
| Humic acid | Humic-like (C) | 237-260/400-500 | 237-260/400-500 |
| Tannic acid | | 260/350 | - |
| Humate | Humic-like (A & C) | 237-260/400-500<br>300-370/400-500 | 237-260/400-500<br>300-370/400-500 |
| Fulvic acid | Humic-like (A & C) | 237-260/400-500<br>300-370/400-500 | 237-260/400-500<br>300-370/400-500 |
| Tyrosine | Protein-like (tyrosine-like) | 225-237/309-321<br>275/310 | 225-237/309-321<br>275/310 |
| Tryptophan | Protein-like (tryptophan - like) | 225-237/340-381<br>275/340 | 225-237/340-381<br>275/340 |
| Microorganism found in distribution pipes† | Protein-like (tyrosine-like) | 225-237/309-321<br>275/310 | 225-237/309-321<br>275/310 |
| Microoragnism found in drinking water catchment‡ | Protein-like (tryptophan - like) | 225-237/340-381<br>275/340 | 225-237/340-381<br>275/340 |
| Proline | | 230/290 | - |

***Note:*** *^Fluorophores identified during experiments; *Fluorophores cited within the literature, see Table 10-1.† See Beale et al., 2010 for more details, microorganism identified to cause microbial influenced corrosion in household plumbing. ‡See Solarska et al., 2009 for more details, microorganism identified within drinking water catchments that degrades NOM.*

### 10.2.1. Application to natural water

To test the effectiveness 3DEEM fluorescence spectrometry, and assist in characterising DOM and pesticide interactions, six water samples collected throughout Victoria, Australia in 2007 (Table 5-3, represented in Table 10-4 for convenience) were pre treated (filtered and adjusted to a pH of 7) prior to 3DEEM analysis. Initial 3DEEM fluorescence spectra from the natural waters sampled indicated a significant variation in DOM characterisation, see Figure 10-5.

**Table 10-4: Summary of characteristics of natural water samples analysed by 3DEEM fluorescence spectrometry.**

| Sample ID | Water Source | DOM | NTU | pH | EC (µS/cm) | $A_{254}$* | Ex/Em λ |
|---|---|---|---|---|---|---|---|
| 1 | Groundwater | 3.1 | 4.3 | 6.0 | 13702 | 0.76 | 310/350<br>235/350 |
| 2 | Creek | 4.5 | 4.1 | 6.9 | 1706 | 0.48 | 290/350<br>225/415 |
| 3 | River | 6.5 | 24.6 | 5.2 | 840 | 0.24 | 300/330<br>225/410 |
| 4 | Drinking water catchment | 11.7 | 1.63 | 7.2 | 447 | 1.1 | 305/350<br>235/350 |
| 5 | Drinking water catchment | 10.7 | 0.65 | 6.9 | 751 | 1.1 | 290/350<br>225/410 |
| 6 | Drinking water catchment | 11.1 | 32.37 | 7.2 | 170 | 1.0 | 300/340<br>225/410 |

*Samples not diluted.

Samples 3 and 6 have strong humic-like (A & C) fluorophores present while sample 2 displays the same fluorophore profile but not as intense. Samples 1, 4 and 5 have strong protein-like fluorophore signatures and a weak humic-like fluorophore present. Similarly, samples 2, 3, and to a lesser extent sample 6, display evidence of protein-like fluorophores. This indicates that all the waters have been exposed to a DOM source that discharges a protein-like fluorophore.

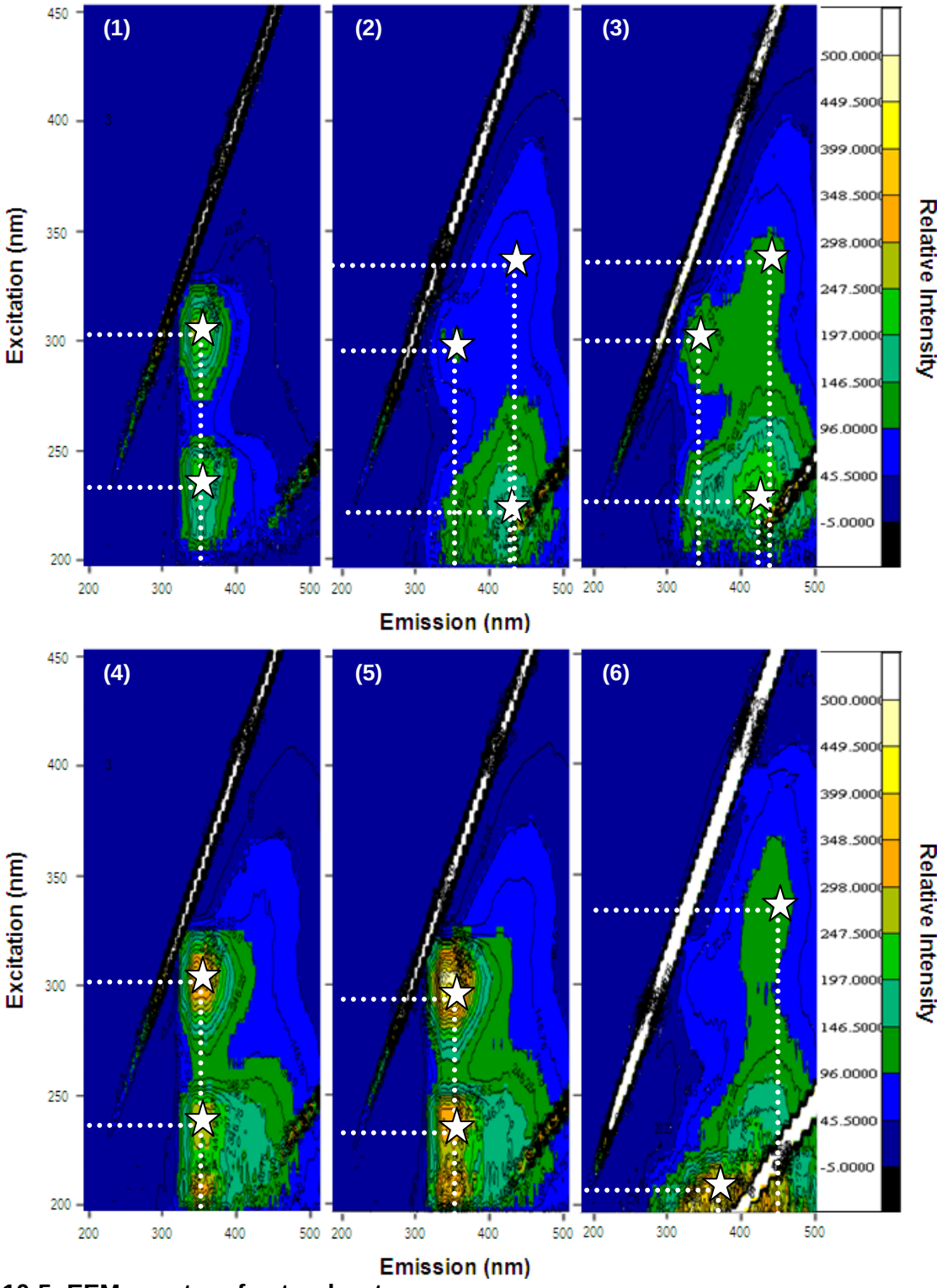



**Figure 10-5: EEM spectra of natural waters**

***NOTE:*** *sample numbers displayed correspond to sample ID numbers in Table 10-4.*

### 10.2.2. Influence of cations and anions

The influence of a selection of cations, anions, and metal ion concentration on the 3DEEM analysis of natural waters was investigated, see Table 10-5.

**Table 10-5: Influence of interfering species on 3DEEM analysis.**

| Species | Sample | ADWG[a] | Change in 3DEEM fingerprint | | |
|---|---|---|---|---|---|
| | | | Humic-like (A) | Humic-like (C) | Protein-like |
| | mg $L^{-1}$ | mg $L^{-1}$ | Δ intensity | Δ intensity | Δ intensity |
| $Ni^{2+}$ | 1.0 | - | <1% | <1% | <1% |
| $Ca^{2+}$ | 1.0 | 200 | <1% | <1% | <1% |
| $Zn^{2+}$ | 1.0 | 3 | <1% | <1% | <1% |
| $Mg^{2+}$ | 1.0 | 0.1 | <1% | <1% | <1% |
| $Cu^{2+}$ | 1.0 | 1 | -100% | -28% | -44% |
| $Cl^{-}$ | 1.0 | 250 | <1% | <1% | <1% |
| $K^{+}$ | 1.0 | - | <1% | <1% | <1% |
| $Al^{3+}$ | 1.0 | 0.2 | 50% | -8% | <1% |
| $Fe^{2+}$ | 1.0 | 0.3 | -100% | -31% | 16% |
| $Fe^{3+}$ | 1.0 | 0.3 | -100% | -44% | 12% |
| Nitrate | 1.0 | 50 | <1% | <1% | <1% |
| Nitrite | 1.0 | 3 | <1% | <1% | <1% |
| $Na^{2+}$ | 1.0 | 180 | <1% | <1% | <1% |

***NOTE:*** *number of replicates, n=3. [a]Australian Drinking Water Guidelines (2004) minimum recommended guideline value for physical and chemical characteristics.*

As illustrated, there was no significant interference from the metal cations or anions with the exception of $Fe^{3+}$, $Fe^{2+}$, and $Cu^{2+}$ which all quenched the humic-like (A & C) fluorophore; $Fe^{3+}$ and $Fe^{2+}$ both enhanced the protein-like fluorophore while $Cu^{2+}$ quenched the fluorophore. $Al^{3+}$ was observed to enhance the humic-like (A) fluorophore while quenching humic-like (C) fluorophore; the protein-like fluorophore was unaffected. The results obtained conflict with some findings found in the literature (as described in Table 10-6), which is most likely due to the non-homogeneous nature of DOM within and between natural environments.

**Table 10-6: Comparison of ion interference with literature findings**

| Ion | Effect on Ex/Em spectra (observed) | Effect on Ex/Em spectra (literature) | Reference |
|---|---|---|---|
| Copper (II) | Blue shift in humic (a) and (b) fluorophore. Reduction in intensity. | Blue-shift in humic-like fluorophore (A)<br>Quenching of humic- and protein-like fluorophores | Wu *et al.*, 2001; Yamashita & Jaffe, 2008; Wang *et al.*, 2009a |
| Iron oxide | Blue shift in proteinlike, humic (a) and (b) fluorophore. Reduction in intensity for humic (A) and (C) fluorophore, increase in intensity for protein-like fluorophore. | Quenching of humic-like fluorophores<br>No observed affect<br>Quenching of humic-like fluorophores | Chen *et al.*, 2003; Pullin *et al.*, 2007; Manciulea *et al.*, 2009 |
| Aluminium | Red shift in humic (C) fluorophore; increase in intensity of humic (A) fluorophore, decrease of intensity of humic (C) fluorophore. | Enhancement Quenching of humic-like fluorophores | Parlanti *et al.*, 2000 |

### 10.2.3. Application for pesticide-DOM complex identification

Huang *et al.* (2008) showed that triazine pesticides can be identified by 3DEEM at high concentrations in manufacturing effluent. In this work, the method was applied to pesticide solutions of much lower concentrations, and proved to be sensitive (*i.e.* 100 µg $L^{-1}$). Figure 10-6 illustrates the EEM fluorescence spectra of triazine, triazinone and organophosphate pesticides (1.0 mg $L^{-1}$) in MilliQ water.

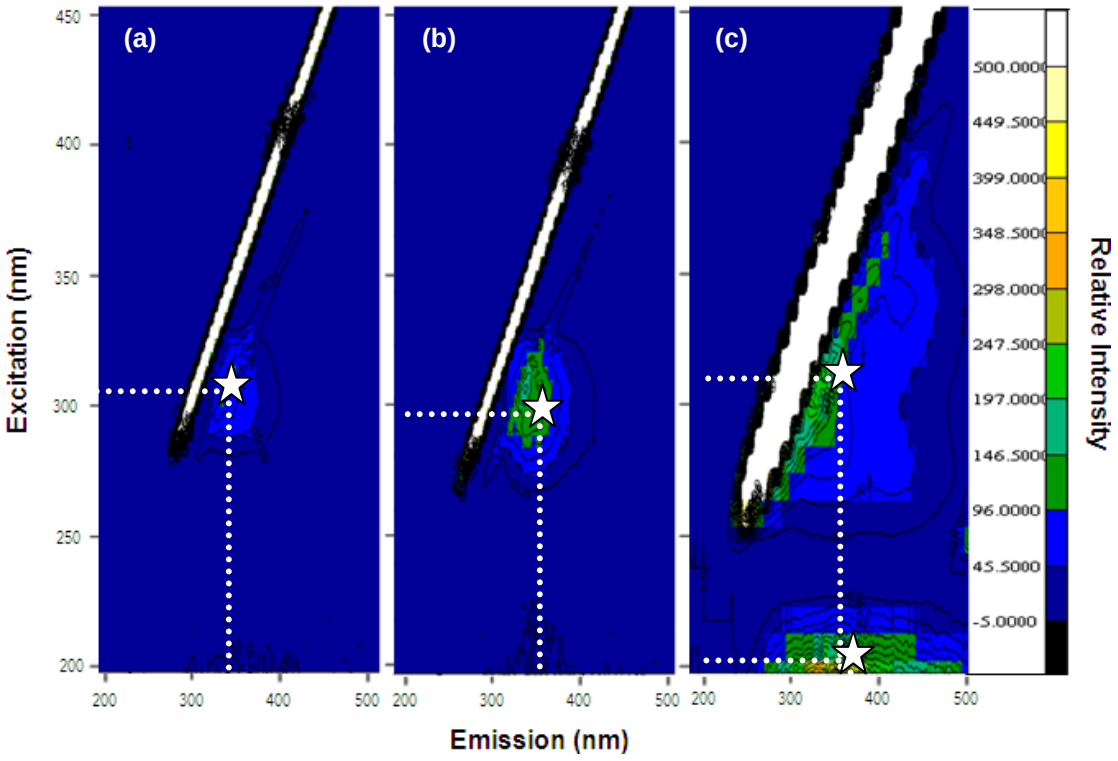



**Figure 10-6: EEM spectra of selected pesticides in MilliQ water**

***NOTE:*** *(a) triazine pesticide (atrazine and simazine) (Ex-310nm; Em-340nm); (b) triazinone pesticide (hexazinone) (Ex-290nm; Em-340nm); and (c) organophosphate (monocrotophos and dicrotophos) (Ex-320nm; Em-340nm, and Ex-205nm; Em-360nm). All solutions were 1 mg $L^{-1}$ in MilliQ water.*

The effect of the pesticides spiked into the natural waters was investigated over the range of 0.001 to 1 mg $L^{-1}$; it was found that concentrations below 0.1 mg $L^{-1}$ had no significant effect on the raw water fluorescence spectra. Figures 7-7, 7-8 and 7-9 illustrate the effect of the pesticides on the humic-like (A), humic-like (C), and protein-like fluorophores respectively at 0.1 mg $L^{-1}$; indicative of a pesticide-DOM complex being formed. As illustrated in the spectrum, it is evident that the addition of pesticide to the natural waters quenched the 'humic-like A' and 'humic-like C' fluorophore while enhancing the 'protein-like' fluorophore. In addition, the triazine pesticide quenched the humic A fluorophore by an additional 22% (quenched a total of 34% of the natural fluorophore) compared to the triazinone and organophosphate pesticides (quenched 12% of the natural fluorophore).

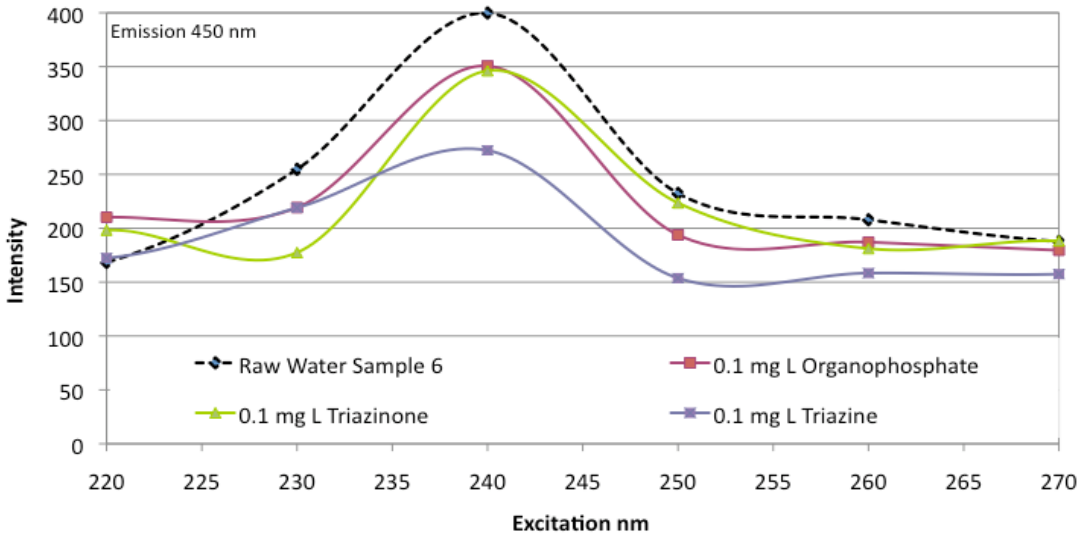



**Figure 10-7: Natural water emission spectra with spiked pesticide (humic-like 'A' fluorophore)**

***Note:*** *Natural water sample (Sample 6) spiked with 0.1 mg $L^{-1}$ pesticide (monocrotophos, an organophosphate; hexazinone, a triazinone; and atrazine, a triazine) and the resulting effect on the humic acid-like (A) fluorophore. Samples were spiked 1 mL in 100 mL (final concentration of 0.1 mg $L^{-1}$ pesticide); raw water sample was spiked with MilliQ water to account for any dilution affect.*

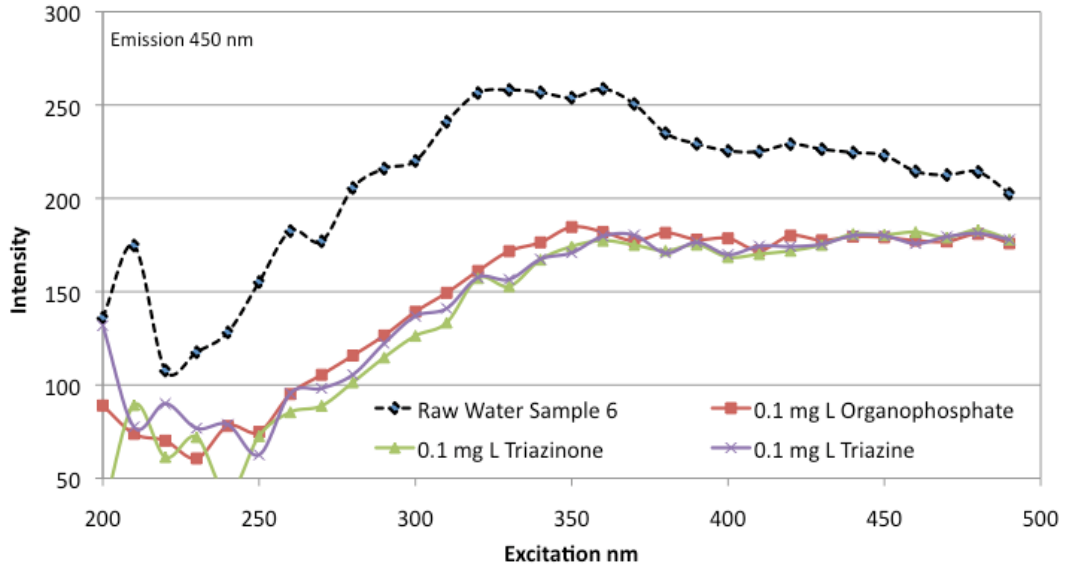



**Figure 10-8: Natural water emission spectra with spiked pesticide (humic-like 'C' fluorophore)**

***Note:*** *Natural water sample (Sample 6) spiked with 0.1 mg $L^{-1}$ pesticide (monocrotophos, an organophosphate; hexazinone, a triazinone; and attrazine, a triazine) and the resulting effect on the humic acid-like (C) fluorophore. Samples were spiked 1 mL in 100 mL (final concentration of 0.1 mg $L^{-1}$ pesticide); raw water sample was spiked with MilliQ water to account for any dilution affect.*

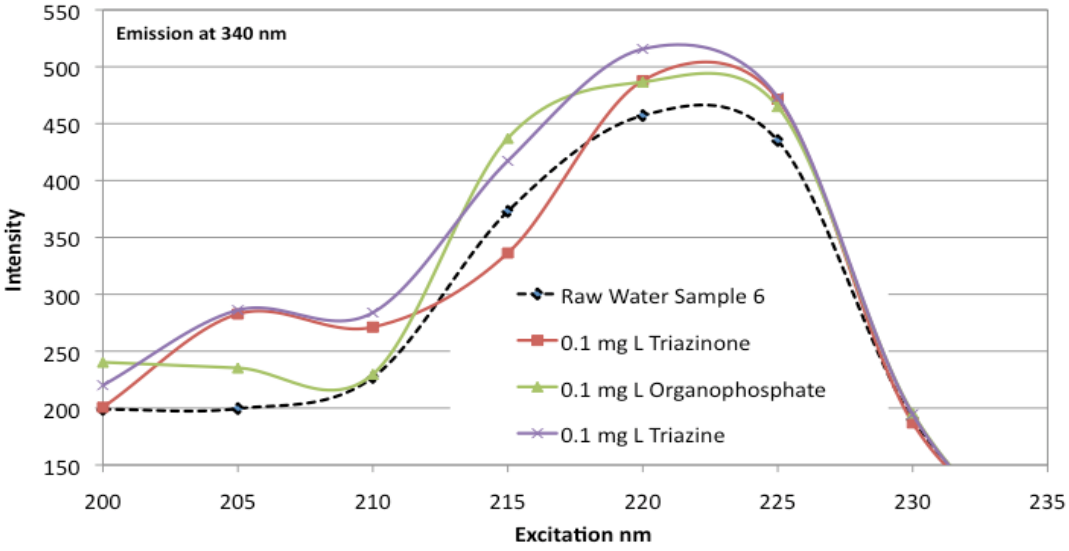



**Figure 10-9: Natural water emission spectra with spiked pesticide (protein fluorophore)**

***Note:*** *Natural water sample (Sample 6) spiked with 0.1 mg $L^{-1}$ pesticide (monocrotophos, an organophosphate; hexazinone, a triazinone; and atrazine, a triazine) and the resulting effect on the protein-like fluorophore. Samples were spiked 1 mL in 100 mL (final concentration of 0.1 mg $L^{-1}$ pesticide); raw water sample was spiked with MilliQ water to account for any dilution affect.*

All the natural samples were analysed to investigate the effect of the pesticide on the DOM, as well as investigating the combination of pesticide, DOM and a known metal ion that affects the fluorescence spectra (see Figure 10-10). As the analysis was a comparison with and without the metal ions and/or pesticides spiked in the natural waters, and while the variation changed in intensity from each sample, the direction of the change (*e.g.*

enhancement or quenching) was consistent; as such, all the samples were aggregated in order to make an assessment of their impact on the 3DEEM fluorescence spectra. Table 10-7 illustrates the enhancement or quenching effect of the pesticides, and the pesticides with a selection of metal ions spiked into the natural waters.

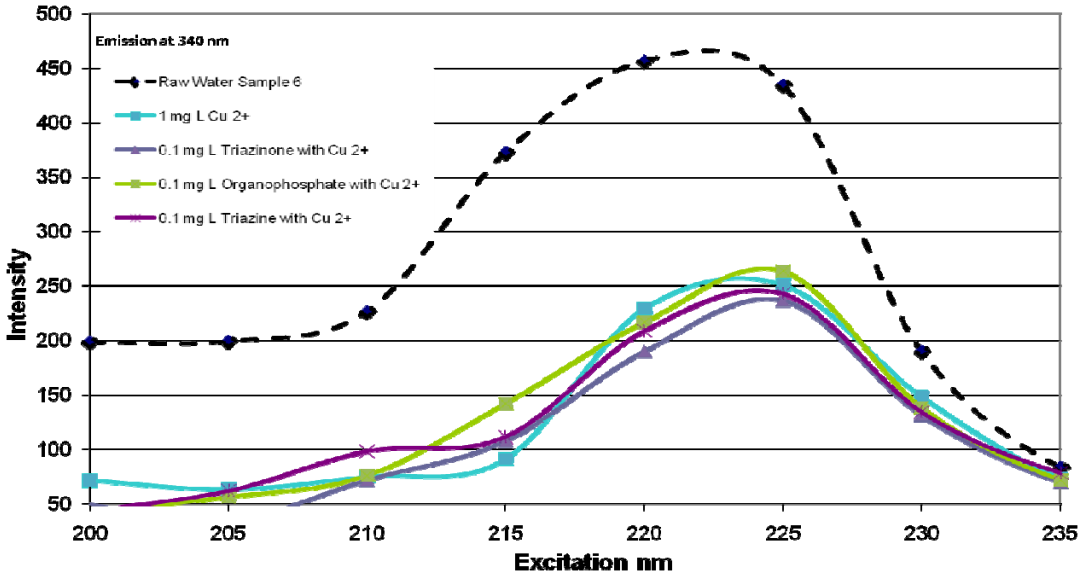



**Figure 10-10: Natural water emission spectra with pesticide and copper**

***Note:*** *Natural water sample (Sample 6) spiked with 0.1 mg $L^{-1}$ pesticide (monocrotophos, an organophosphate; hexazinone, a triazinone; and atrazine, a triazine) and 1.0 mg $L^{-1}$ $Cu^{2+}$ and the resulting effect on the protein-like fluorophore. Samples were spiked 1 mL in 100 mL; raw water sample was spiked with MilliQ water to account for any dilution affect.*

**Table 10-7: Effect on DOM 3DEEM spectra caused by pesticides, and pesticides with a selection of metal ions.**

| Species | 3DEEM mg $L^{-1}$ | ADWG[a] mg $L^{-1}$ | Humic-like (A) Δ intensity (±%) | Humic-like (C) Δ intensity (±%) | Protein-like Δ intensity (±%) |
|---|---|---|---|---|---|
| **Triazine pesticides** | | | **-42.5% (1.2)** | **-41.2% (3.5)** | **8.6% (7.1)** |
| $Cu^{2+}$ | 1.0 | 1 | -100% (<5) | -28% (4.7) | -46.2% (4.8) |
| $Fe^{2+}$ | 1.0 | 0.3 | -100% (<5) | -31% (6.2) | -100% (<5) |
| $Fe^{3+}$ | 1.0 | 0.3 | -100% (<5) | -44% (5.5) | 10.5%(2.4) |
| $Al^{3+}$ | 1.0 | 0.2 | 50% | -27% (4.0) | <1% (<5) |
| **Triazinone pesticides** | | | **-12.5% (5.8)** | **-40% (6.5)** | **11.8% (3.3)** |
| $Cu^{2+}$ | 1.0 | 1 | -100% (<5) | -28% (6.5) | -48.4% (7.5) |
| $Fe^{2+}$ | 1.0 | 0.3 | -100% (<5) | -31% (7.3) | -100% (<5) |
| $Fe^{3+}$ | 1.0 | 0.3 | -100% (<5) | -44% (4.0) | -100% (<5) |
| $Al^{3+}$ | 1.0 | 0.2 | 60% (8.4) | -30% (7.1) | <1% (<5) |
| **Organophosphate pesticides** | | | **-13% (3.2)** | **-43.5% (7.5)** | **5.4% (1.2)** |
| $Cu^{2+}$ | 1.0 | 1 | -100% (<5) | -28% (8.7) | -45.2% (6.4) |
| $Fe^{2+}$ | 1.0 | 0.3 | -100% (<5) | -31% (8.2) | -100% (<5) |
| $Fe^{3+}$ | 1.0 | 0.3 | -100% (<5) | -44% (10.8) | 10.6% (1.1) |
| $Al^{3+}$ | 1.0 | 0.2 | 60% (6.8) | -34% (7.1) | <1% (<5) |
| **Pesticide free** | | | **-13% (3.2)** | **-43.5% (7.5)** | **5.4% (1.2)** |
| $Cu^{2+}$ | 1.0 | 1 | -100% (<5) | -28% (<5) | -44% (<5) |
| $Fe^{2+}$ | 1.0 | 0.3 | -100% (<5) | -31% (<5) | 16% (<5) |
| $Fe^{3+}$ | 1.0 | 0.3 | -100% (<5) | -44% (<5) | 12% (<5) |
| $Al^{3+}$ | 1.0 | 0.2 | 50% (<5) | -8% (<5) | <1% (<5) |

Table 10-8 summarises the effects of adding of pesticides, metal ions and the combination of the two on the observed DOM fingerprint.

**Table 10-8: Effect on DOM 3DEEM spectra caused pesticides, and pesticides with a selection of metal ions.**

| Sample/standard | Metal ion effect on fluorophore | Pesticide effect on fluorophore | Pesticide with a Metal ion effect on fluorophore |
|---|---|---|---|
| Protein-like | $Fe^{2+}$ 'blue' shift, $Al^{3+}$ increased intensity. | All intensities increase | $Al^{3+}$ and $Fe^{2+}$ intensity increase. |
| Humic-like (A) | $Al^{3+}$ intensity increase. | All intensities increase. | $Al^{3+}$ intensity increase. |
| Humic-like (C) | $Al^{3+}$ 'red' shift. | All intensities increase. | $Al^{3+}$ shift and increase |
| Natural samples | Red shift Al, Blue shift for $Cu^{2+}$ and $Fe^{2+}$ | Red shift and increase | Red shift and decrease for Al; Blue shift and decrease for $Cu^{2+}$ and $Fe^{2+}$ |

As a result of understanding the characterisation of the DOM within the waters sampled, it is apparent that the analysis of natural waters by 3DEEM fluorescence is sample specific; while the presence of these pesticides can be identified through variations in fluorescent

intensities (*e.g.* of humic-like (A and C) fluorophores) it is difficult to state with any real confidence that any observed change is a result of a specific contaminant such as atrazine. Also, any observed change may simply be the result of environmental variation (*e.g.* DOM fluctuations, desorbing and adsorbing of metals etc.). To overcome this, it would be ideal to monitor the fingerprint of a water source over a long time frame (*e.g.* several years), where trends can be established and significant variations can be tracked and monitored through more robust analytical techniques (*e.g.* GC-MS).

It is also apparent from Table 10-10, that there is an interaction between the pesticides analysed (grouped as triazines, triazinone and organophosphate) with the DOM in natural waters, specifically at the humic-like (A) and (C) fluorophore. This would suggest that when extracting target analytes from the natural samples in order to eliminate the interference observed by DOM in FICA, there is potential to lose analyte sensitivity due to pesticide bound to the DOM. This was evident when investigated in Chapter Eight, extracting target analyte(s) from natural waters.

## 10.3. Conclusions

3DEEM fluorescence spectroscopy was applied to natural water samples to characterise the DOM, and to investigate the interaction between DOM and the selected pesticides. Subsequent comparisons of known fluorophore standards and natural waters were used to identify the variety of DOM signatures in the selected samples.

Upon further investigation into the effects of pesticides and metal interactions, it was observed that all three classes of pesticides inhibited the fluorescence intensity at the humic-like (A) and (C) fluorophore, and in the presence of Fe, Al and Cu were observed to shift or increase the fluorophore intensity. This did not correlate with experiments investigating metals in isolation (*i.e.,* without pesticide).

It is concluded that while fingerprint analysis of natural waters can be done to identify the presence of contaminants, it requires the analysis to be done over a large temporal scale to establish if the observed effect is due to seasonal variations or the result of a contamination event (which may or may not be due to pesticide contamination).

# CHAPTER ELEVEN: CONCLUSIONS, RECOMMENDATIONS AND FUTURE RESEARCH

This chapter presents the many conclusions which can be drawn from this work, along with an outline of recommendations and future work which will hopefully be addressed to further the development of the proposed instrument and its application to pesticide residue monitoring in natural waters.

## 11.1. Conclusions

Review of the available literature has highlighted community awareness on the observed and potential impacts of pesticide use and exposure, and there is an observed increase in the scrutiny that is being applied to government agencies that control and regulate their use and the commercial entities that rely on them.

The monitoring and analysis of pesticides within the potable water sector is an expensive and daunting task for most water companies. The number of possible contaminants (both chemical and biological) that can enter into the water supply is very large and in areas of intense agriculture concentrations can be significantly higher (*e.g.* in the magnitude of mg $L^{-1}$). Given the level of scrutiny the water sector operates under (namely water quality, water availability and pricing), techniques such as flow injection chemiluminescence analysis (FICA) are needed for the determination of pesticide residues in source waters and water within the distribution system. From the literature, it was identified that the pesticides atrazine, simazine and hexazinone have either a history of contamination or have the potential to contaminate water ways. As such, these pesticides were selected for investigation to gauge there suitability for FICA. In addition, monocrotophos and dicrotophos were selected for inclusion in the study based on their structure including an aliphatic amine moiety.

The FICA method presented utilises chemically oxidized chemiluminescent reagents, tris(2,2'-bipyridyl)ruthenium(III) and luminol, which have been successfully applied for the determination of compounds comprising an aliphatic amine moiety and organophosphates, respectively. A multivariate and univariate optimisation method was applied. The optimised tris(2,2'-bipyridyl)ruthenium(III) experimental conditions were: sample and carrier flow rates of 4.6 mL $min^{-1}$, sample at pH 9 buffered with 50 mM borax, and a reagent concentration of

1 mM tris(2,2'-bipyridyl)ruthenium(III) in 20 mM $H_2SO_4$ (pH 1). The developed optimised luminol experimental conditions for monocrotophos and dicrotophos were determined to be: sample and carrier flow rates of 3.0 mL $min^{-1}$, sample at pH 9 buffered with 50 mM borax, and a reagent concentration of 2.75 mM luminol reagent in 0.1 M NaOH.

Once the operating conditions were defined, a series of experiments was carried out in order to further enhance the capabilities of the instrument and reduce the limits of detection to below ADWG. The experiments comprised: analysis of pesticide residues in MilliQ water; analysis of pesticide residues in natural waters; analysis of pesticide residues with in-line SPE; and, analysis of multiple pesticides residues with in-line SPE and monolithic separation.

In Chapter Four, the analysis of pesticide residues in MilliQ water is presented where atrazine was detected using tris(2,2'-bipyridyl)ruthenium(III) chemiluminescence with a limit of detection of 1.3 ± 0.1 µg $L^{-1}$. Validation of the method was performed by direct injection HPLC, with no significant difference observed between the methods. ($R^2$ = 0.9906, t-test (6) = 0.39 (p two tailed = 0.71) and -0.74 (p two tailed = 0.48) for 0.5 and 10 µg $L^{-1}$ atrazine respectively). The HPLC method was further developed in Chapter Five by incorporating a monolithic column which significantly decreased the analysis time.

Atrazine, simazine, hexazinone, monocrotophos and dicrotophos were detected within 2 minutes for each sample by modified large volume direct injection HPLC with a limit of detection of 0.5, 0.6, 1.0, 2.0 and 0.2 µg $L^{-1}$, respectively, in milliQ water without pre-concentration. Validation of the method was performed using a series of analytical standards, with good correlation achieved with samples spiked at 10 and 50 µg $L^{-1}$ for atrazine, simazine and hexazinone.

Analysis of natural waters comprising various concentrations of natural organic matter (DOM; 3.1 – 11.7 mg $L^{-1}$) had no significant effect on the resolution or separation capacity for atrazine, simazine and hexazinone. Monocrotophos and dicrotophos were deemed unsuitable for this analysis since both analytes co-eluted with the DOM peak.

It was shown in HPLC and with a monolithic column that 'like' compounds, such as other triazines and atrazine metabolites can be differentiated from the target analytes. However, it was found that atrazine-2-hydroxy has the potential to co-elute with simazine under the described operating conditions.

While the application FICA was successful when analysing atrazine in clean samples, when applied to natural waters (Chapter Seven), the presence of DOM caused a significant positive chemiluminescent response which masks the signal from atrazine. The functional groups responsible for the interference were identified by ATR-FTIR as amines and hydroxyl groups present in the natural water. In addition, the effect of various cations and anions was investigated at levels common in natural waters. It was observed that $Fe^{3+}$ and $Fe^{2+}$ (at concentrations above ADWG) caused interference. It was also shown that similar compounds, such as the atrazine metabolites and other triazine pesticides, produced a chemiluminescent signal with tris(2,2-bipyridyl)ruthenium(III). However, the interference from DOM was removed by SPE. As a result, the detection limit for atrazine in natural water samples to 14 ± 2 ng $L^{-1}$

As a side, the evaluation of luminol and tris(2'2-bipridyl)ruthenium(III) chemiluminescence was evaluated for the determination of monocrotophos and dicrotophos (presented in Chapter Six). Here luminol was successfully applied to the detection of dicrotophos (LOD 18.1 µg L-1) and monocrotophos (LOD 7.1 µg $L^{-1}$) in MilliQ water and natural water samples containing DOM. Chemiluminescence generated using luminol was found to be better than with tris(2'2-bipridyl)ruthenium(III) for the selected organophosphates because of its greater sensitivity and freedom from interference. While the detection limit was above the current health trigger value set in the ADWG, it could be further reduced using online extraction and pre concentration.

The incorporation of an in-line extraction column presented in Chapter Eight enabled the rapid detection of pesticide residues that had previously proven to be difficult due to interfering species. A variety of extraction resins were evaluated, namely: MIEX© (used to remove DOM), C18 and Nexus© (used to trap target analytes). It was found that Nexus© increased the capacity for larger extractions to be undertaken due to the multi-layer absorption capacity of the resin. This allowed the rapid analysis of smaller volumes (*i.e.,* 100 mL) to be carried out without an off-line extraction step (which can be time consuming and consumes larger volumes of solvent). The analysis of atrazine, hexazinone, and simazine by in-line SPE (with Nexus©) was successfully applied with method detection limits of 14, 48 and 32 ng $L^{-1}$, respectively. No positive interferences were observed.

Lastly, the benefits of the monolithic column described in Chapter Five were merged with the advantages of the in-line SPE outlined in Chapter Eight, creating a hybrid FICA system

analogous to a low pressure HPLC system (presented in Chapter Nine). The incorporation of a monolithic column enabled atrazine, simazine and hexazinone to be detected simultaneously with chromatographic differentiation, with method detection limits of 27, 39 and 60, respectively. Upon further investigation, it was observed that atrazine metabolites atrazine-2-hydroxy, atrazine-desisopropyl, and atrazine-desethyl would cause interference if in solution.

In Chapter Ten, 3DEEM fluorescence spectroscopy was applied to natural water samples to characterise the DOM, and to investigate the interaction between DOM and the selected pesticides. It was observed that all three classes of pesticides inhibited the fluorescence intensity at the humic-like (A) and (C) fluorophore, and in the presence of Fe, Al and Cu were observed to shift or increase the fluorophore intensity. It is concluded a pesticide-natural organic matter complex was being formed. It should be noted, that while fingerprint analysis of natural waters can be done to identify the presence of contaminants, it requires the analysis to be done over a large temporal scale to establish if the observed effect is due to seasonal variations or the result of a contamination event (which may or may not be due to pesticide contamination).

Overall, the FICA system described will be very useful as a quick, sensitive screening method for atrazine, simazine, hexazinone and selected metabolites in natural waters. The methods developed in this study should be considered by water utilities for inclusion in their ongoing pesticide monitoring programs, and with further refinement and enhancement they could be used on site eliminating the need to remove samples for analysis. The FICA system described with the in-line SPE and monolithic separation presents a low cost solution for the rapid analysis of pesticide residues in natural waters. While it was observed that the tris(2'2-bipridyl)ruthenium(III) reagent reacted with DOM in natural waters, this interference was overcome with the addition of a in-line SPE cartridge. In addition, the use of the tris(2'2-bipridyl)ruthenium(III) reagent in a portable FICA system was found to be limited. As a general rule, fresh reagent was prepared each day in order to ensure reproducible analysis of samples due the limited working life of the reagent. As such, tris(2'2-bipridyl)ruthenium(III) chemiluminescence is a suitable reagent for a portable FICA system that can be refreshed daily or as required. However, its application for an in-situ instrument requiring operation over a pro-longed period (e.g. more than a week) would be limited.

Through FICA, a water utility would be able to increase the frequency of analysis as well as the number of samples analysed. Within this context, the application would enable water utilities to screen a larger number of samples, with only samples that provide a positive chemiluminescence signal being confirmed using standard analytical approaches (*i.e.,* GC-MS and LC-MS).

## 11.2. Recommendations

There were a number of issues that arose during the conduct of this research project, which due to time restraints and diminished project funds require further investigation. These include:

1. Investigating gradient elution in the FICA system with in-line SPE and monolithic separation. While the multiple analyte detection concept has been demonstrated using an isocratic mobile phase, the effect of interfering 'like' compounds (such as atrazine metabolites) could be removed though gradient elution. This would not only have the potential of increasing the number of analytes that can be detected at once but also raise the confidence of the analyst by discriminating between more compounds.
2. The multi-component aspect of the FICA system with in-line SPE and monolithic separation potentially could be streamlined by reducing the number of multiple pumps continuously flowing and eliminating the multiple merging points. This can be achieved by changing the current flow through system to a sequential system known as sequential injection analysis (discussed in Chapter Eight).
3. In addition to point 2, utilising a lab-on-a-valve (LOV) manifold (FIAlab, USA) in a SIA set up of the described FICA system could increase the overall sensitivity of the instrument, further reducing detection limits. The LOV manifold has a built-in flow cell which has the ability to capture the chemiluminescence reaction via a fibre optic cable as the reagent and sample streams merge; ensuring all of the reaction from the point of mixing is captured.
4. A LOV manifold would also facilitate the removal of the in-line extraction column since the manifold can facilitate micro extractions on-board. By undertaking the extraction process on-board the manifold, the overall pressure of the system will be reduced. Extractions undertaken on a LOV manifold comprise a purpose built

extraction chamber that contains the extraction resin within a free flowing chamber (*i.e.,* the resins is not densely packed). The addition of a free flowing extraction resins enables the operator more flexibility in the terms of the size of extraction and where within the manifold the extraction takes place, as the resins can be easily transferred throughout the manifold, and extraction fractions withheld.

5. Lastly, the reconfiguration from a flow through system to a sequential one incorporating a LOV manifold would reduce the overall power requirements of the instrument (as the number of pumps would be reduced). Such a reduction would facilitate the transfer from a desktop instrument (which it is currently) into a fully portable field instrument.

## 11.3. Future Research

In addition to the application of the described FICA instrument to natural waters within catchments and distribution systems, the instrument could be adapted for use in other environments. These include:

1. Investigate the suitability of FICA to other pesticides. Mesotrione is a herbicide that is slowly being used in place of atrazine. However, the impact of mesotrione onto the environment is not yet fully realised. The USEPA is currently supporting experimental toxicology and mobility studies to collect such data. It should be noted, the application the FICA instrument described has the potential to be used for the determination of a range of other pesticides; mesotrione is but one possible pesticide and due to the limited information on its application to agriculture, it could be a pesticide of concern in the near future.

2. Many chemicals such as metham sodium and trifluralin have been used to control tree root invasion in sewer networks (Ware, 2000). Chemical treatments can be done alone or in combination with mechanical techniques (*e.g.* root cutting). In either case, the modes of delivering chemical treatments include soaking (isolating the sewer and filling it with a chemical treatment), foaming (the chemical is applied as expansive foam) or spot spraying (the chemical is applied directly onto the roots in the sewer via a CCTV inspection device rigged with a spraying apparatus). However, there are some important negative impacts associated with the use of such chemicals. These include adverse effects on wastewater treatment plant operation

(through toxicity to bacterial nitrifiers), unintended damage to downstream waterways and ecosystems where treated wastewater is discharged. The application of FICA to sewer waters could enable water utility operators better manage and monitor pesticide applications within sewers for the control of tree roots. However, it is noted, a stronger emphasis would be needed on the sample extraction and cleanup step to ensure the continual successful operation of the FICA system. In addition, a preliminary study would need to be carried out on the feasibility of detecting metham sodium and trifluralin by FICA.

3. As illustrated by Benotti *et al.* (2009), pesticides and other organic chemicals of concern are routinely being detected in treated waste waters after secondary and tertiary treatment. With an increased focus on recycled water globally (either for drinking or re-use via a third pipe system), a FICA instrument that enables rapid analysis of target pesticides after treatment can be used, offering the same benefits as its application in natural waters. It would enable more samples to analysed and increase the frequency of analysis. Likewise, positive samples would need to be confirmed by more traditional techniques. Such a tool could be used to increase consumer confidence and uptake.

4. Apart from further improvements to the FICA system, investigating the temporal fluorescence signatures of drinking water catchments to identify changes, sources of contamination, and seasonal fluctuations on natural organic matter may be a useful tool for a water catchment authority. A chemometric analysis could then be used to identify trends and anomalies to catchment waters, in conjunction with detailed analytical profiling of the waters focusing on organic synthesized contaminates, metabolites and olfactory compounds that may cause water quality complaints and breaches at consumer taps.

# APPENDIX A: PESTICIDE CONTAMINATION IN AUSTRALIAN WATERWAYS

| Pesticide Name | Water Body | Year | Application | Location | State | Detected Level (μg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| Hexazinone | Surface Waters | 2007 | Catchment | Moorabool WTP | Victoria | 0.06 - 0.13 | Amis, 2008 |
| Atrazine | Surface Waters | 2006 | Catchment | Central Goulburn Irrigation | Victoria | 0.04 - 0.086 | Amis, 2008) |
| Atrazine | Surface Waters | 2006 | Catchment | Mildura | Victoria | 0.03 | Amis, 2008 |
| Hexazinone | Surface Waters | 2006 | Catchment | Moorabool WTP | Victoria | 0.032 - 0.17 | Amis, 2008 |
| Atrazine | Surface Waters | 2006 | Catchment | Piangil Supply | Victoria | 0.03 | Amis, 2008 |
| Atrazine | Surface Waters | 2006 | Catchment | Red Cliffs Supply | Victoria | 0.03 | Amis, 2008 |
| Atrazine | Surface Waters | 2006 | Catchment | Rochester | Victoria | 0.02 - 0.039 | Amis, 2008 |
| Hexazinone | Surface Waters | 2006 | Catchment | Stony Creek Reservoir | Victoria | 0.084 - 0.20 | Amis, 2008 |
| Atrazine | Surface Waters | 2006 | Catchment | Torrumbarry Irrigation Area | Victoria | 0.031 - 0.050 | Amis, 2008 |
| Simazine | Irrigation | 2005 | Agriculture | Murrumbidgee | New South Wales | 17.8 | Tran *et al.*, 2007 |
| Atrazine | Irrigation | 2005 | Agriculture | Murrumbidgee | New South Wales | 0.9 | Tran et al., 2007 |
| Atrazine | Ocean | 2005 | Great Barrier Reef | North | Queensland | ng $L^{-1}$ range | Shaw & Muller, 2005 |
| Hexazinone | Ocean | 2005 | Great Barrier Reef | North | Queensland | ng $L^{-1}$ range | Shaw & Muller, 2005 |
| Simazine | Ocean | 2005 | Great Barrier Reef | North | Queensland | ng $L^{-1}$ range | Shaw & Muller, 2005 |
| Hexazinone | Surface Waters | 2005 | Catchment | Korweinguboora Inlet | Victoria | 0.051 - 0.86 | Amis, 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Bannockburn Basin | Victoria | 0.19 | Amis, 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Bostok Outlet | Victoria | 0.44 - 0.58 | Amis, 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Bungal Creek | Victoria | 0.047 - 0.082 | Amis , 2008 |
| Atrazine | Surface Waters | 2005 | Catchment | Central Goulburn Irrigation | Victoria | 0.01 - 0.025 | Amis, 2008 |
| Atrazine | Surface Waters | 2005 | Catchment | Irrigation Area | Victoria | 0.024 | Amis, 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Korweinguboora Inlet | Victoria | 0.37 - 1.3 | Amis, 2008 |

| Pesticide Name | Water Body | Year | Application | Location | State | Detected Level (μg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| Hexazinone | Surface Waters | 2005 | Catchment | Korweinguboora Outlet | Victoria | 0.2 - 9.4 | Amis, 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Montpellier Basin | Victoria | 0.1 | Amis, 2008 |
| Atrazine | Surface Waters | 2005 | Catchment | Murray Valley Irrigation Area | Victoria | 0.017 - 0.056 | Amis, 2008 |
| Atrazine | Surface Waters | 2005 | Catchment | Pyramid Boort Irrigation Area | Victoria | 0.01 - 0.032 | Amis, 2008 |
| Atrazine | Surface Waters | 2005 | Catchment | Rochester | Victoria | 0.011 - 0.017 | Amis , 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Stony Creek Reservoir | Victoria | 0.16 - 0.24 | Amis, 2008 |
| Atrazine | Surface Waters | 2005 | Catchment | Torrumbarry Irrigation Area | Victoria | 0.022 - 0.035 | Amis, 2008 |
| Hexazinone | Surface Waters | 2005 | Catchment | Upper Stony Creek Reservoir | Victoria | 0.15 - 0.21 | Amis, 2008 |
| Atrazine | Surface Waters | 2004 | Oyster farms | 90% mortality | Tasmania | | Radcliff, 2002 |
| Atrazine | Surface Waters | 2004 | Catchment | Pyramid Boort Irrigation Area | Victoria | 0.01 - 0.033 | Amis, 2008 |
| Hexazinone | Surface Waters | 2004 | Catchment | Stony Creek Reservoir | Victoria | 0.32 | Amis, 2008 |
| Atrazine | Surface Waters | 2004 | Catchment | Torrumbarry Irrigation Area | Victoria | 0.01 - 0.028 | Amis, 2008 |
| Atrazine | Streams | 2003 | | Whitsundays | Queensland | 1.3 | Mitchell et al., 2005 |
| Hexazinone | Streams | 2003 | | Whitsundays | Queensland | 0.3 | Mitchell et al., 2005 |
| Simazine | Surface Waters | 2002 | Catchment | Rocky River | Victoria | 0.2 | Amis, 2008 |
| Atrazine | Surface Waters | 2000 | | North-Western | New South Wales | <20,000 | Radcliff, 2002 |
| Atrazine | Streams | 2000 | | Condamin-Bolonne river system | Queensland | <2,400 | Radcliff, 2002 |
| Atrazine | Streams | 2000 | Forestry | | Western Australia | 800-2,400 | Radcliff, 2002 |
| Atrazine | Irrigation (sediment) | 2000 | Cotton/Sugar Cane | Coastal | Queensland | <0.013 | Muller *et al.*, 2000 |
| Simazine | Irrigation (sediment) | 2000 | Cotton/Sugar Cane | Coastal | Queensland | <0.00061 | Muller *et al.*, 2000 |

| Pesticide Name | Water Body | Year | Application | Location | State | Detected Level (μg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| Hexazinone | Irrigation (sediment) | 2000 | Cotton/Sugar Cane | Coastal | Queensland | <000038 | Muller *et al.*, 2000 |
| Atrazine | Irrigation (sediment) | 2000 | | MacKay | Queensland | 0.3 | Haynes et al. , 2000 |
| Atrazine | Surface Waters | 1999 | Forestry | | Tasmania | 0.2 | Barnes & Holz, , 1999 |
| Atrazine | Groundwater | 1996 | Agriculture | Shepparton East | Victoria | Not specified | Kookana et al., 1998 |
| Atrazine | Groundwater | 1996 | Irrigation | Burdekin River | Queensland | Not specified | Kookana et al. , 1998 |
| Atrazine | Groundwater | 1996 | Town supply | Bundaberg | Queensland | Not specified | Kookana et al., 1998 |
| Atrazine | Groundwater | 1995 | Bore Wells | Padthaway | South Australia | 60 | Radcliff, 2002 |
| Simazine | Groundwater | 1995 | Bore Wells | Padthaway | South Australia | 65 | Radcliff, 2002 |
| Atrazine | Surface Waters | 1995 | | Padthaway | South Australia | 4-600 | Radcliff, 2002 |
| Simazine | Surface Waters | 1995 | | Padthaway | South Australia | 4-600 | Radcliff, 2002 |
| Atrazine | Irrigation | 1995 | Maize | | New South Wales | 145 | Korth, 1995 |
| Atrazine | Streams | 1994 | | Orbost | Victoria | <4,900 | Radcliff, 2002 |
| Simazine | Streams | 1994 | | Orbost | Victoria | <1900 | Radcliff, 2002 |
| Atrazine | Streams | 1994 | | Rosebud | Victoria | <140 | Radcliff, 2002 |
| Atrazine | Streams | 1994 | | Bairnsdale | Victoria | <3,200 | Radcliff, 2002 |
| Hexazinone | Streams | 1994 | Catchment | Gippsland | Victoria | <2 | Radcliff, 2002 |
| Atrazine | Groundwater | 1994 | Bore Wells | Orbost | Victoria | 60 | Radcliff, 2002 |
| Atrazine | Irrigation | 1994 | Rice Crops | Murrumbidgee | New South Wales | Not specified | Kookana et al., 1998 |
| Atrazine | Groundwater | 1993 | Bore Wells | Atherton tableland | Queensland | 1,400 | Radcliff, 2002 |
| Hexazinone | Groundwater | 1993 | Bore Wells | Atherton tableland | Queensland | 100 | Radcliff, 2002 |
| Atrazine | Streams | 1993 | Cotton Farms | Condamin-Bolonne river system | Queensland | Not specified | Kookana et al., 1998 |

| Pesticide Name | Water Body | Year | Application | Location | State | Detected Level (μg $L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| Atrazine | Irrigation | 1993 | Agriculture | Darling Downs | Queensland | Not specified | Kookana et al., 1998 |
| Atrazine | Groundwater | 1993 | Agriculture | South-east | South Australia | Not specified | Kookana et al., 1998 |
| Atrazine | Streams | 1992 | Forestry | 20/29 Streams | Tasmania | <53,000 | Davies *et al.*, 1994 |
| Simazine | Streams | 1992 | Forestry | 20/29 Streams | Tasmania | <478 | Radcliff, 2002 |
| Atrazine (plus metabolites) and Simazine | Groundwater | 1992 | Bore Wells | 80% of samples | Australia | <2 | Radcliff, 2002 |
| Simazine | Groundwater | 1992 | Bore Wells | Coastal plain | New South Wales | 10 | Radcliff, 2002 |
| Atrazine | Groundwater | 1992 | Bore Wells | Coastal plain | New South Wales | <5,800 | Radcliff, 2002 |
| Atrazine and Simazine | Groundwater | 1992 | Bore Wells | Shepparton | Victoria | 60 | Radcliff, 2002 |
| Atrazine and Simazine | Groundwater | 1992 | Bore Wells | Nagambie | Victoria | 950 | Radcliff, 2002 |
| Atrazine | Groundwater | 1992 | Agriculture | Shepparton East | Victoria | Not specified | Kookana et al., 1998 |
| Atrazine (plus metabolites) | Surface waters | 1991 | Cotton Farms | Namoi | New South Wales | <2,250 | Radcliff, 2002 |
| Atrazine | Groundwater | 1991 | Urban use | Ord River | Western Australia | Not specified | Kookana et al., 1998 |
| Atrazine | Groundwater | 1991 | Point source | Perth | Western Australia | Not specified | Kookana et al., 1998 |
| Atrazine | Streams | 1966 | - | Daintree River | Queensland | Not specified | Kookana et al., 1998 |
| Atrazine | Irrigation | 1966 | Cotton Farms | Gwydir River | New South Wales | Not specified | Kookana et al., 1998 |
| Atrazine | Irrigation | 1966 | Cotton Farms | Border River | New South Wales | Not specified | Kookana et al., 1998 |
| Atrazine | Irrigation | | Rice Crops | South-western | New South Wales | <200 | Radcliff, 2002 |

# APPENDIX B: TRIAZINE EXPOSURE ON SELECTED SPECIES

| Common name | Sub species | Year | Age | Triazine | Minimum Exposure µg $L^{-1}$ | Maximum Exposure µg $L^{-1}$ | Experiment timeframe (days) | Observed effects | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Test species with no significant observed affects from pesticide exposure** | | | | | | | | | |
| African Clawed Frog | *X. laevis* | 2002 | Juvenile | Atrazine | 1 | 25 | 300 | The presence of testicular oocytes was not related to exposure to atrazine and may be a natural phenomenon. | Jooste et al., 2005. |
| African Clawed Frog | *X. laevis* | 2005 | Juvenile | Atrazine | 0.1 | 25 | 100 | No significant affect on mortality, growth, gonad development, laryngeal muscle size, or aromatase activity | Coady *et al.* , 2005. |
| African Clawed Frog | *X. laevis* | 2008 | Embryo - Adults | Atrazine | 1 | 25 | 730 | No significant affects to reproductive fitness and development of frogs observed. | Du Preez *et al.*, 2008. |
| Atlantic Salmon | *Salmo salar L.* | 2007 | Juvenile | Hexazinone | | 100 | 21 | No observed side effects | Nieves-Puigdoller *et al.*, 2007 |
| Gold fish | *Carassius auratus L.* | 2008 | Juvenile | Atrazine | 100 | 1000 | 56 | No observed side effects | Nadzialek *et al.*, 2008. |
| Interstitial crustaceans | *Crustacea Mysidacea* | 2007 | Juvenile | Atrazine | 0.01 | 500000 | 21 | 100% mortality at high concentration - no observed effects at environmental levels (<1 g $L^{-1}$) | Noppe *et al.*, 2007. |

| Common name | Sub species | Year | Age | Triazine | Minimum Exposure µg $L^{-1}$ | Maximum Exposure µg $L^{-1}$ | Experiment timeframe (days) | Observed effects | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Japanese Quail | *Coturnix coturnix japonica* | 2005 | Male Adults | Atrazine | 10000 | 1000000 | - | Atrazine administered systemically exerted no effect on indices of growth or reproduction | Wilhelms *et al.*, 2005. |
| Japanese Quail | *Coturnix coturnix japonica* | 2006 | Female Adolescent | Atrazine | 1 | 1000000 | - | No adverse effects - lack of general or reproductive toxicity in birds | Wilhelms *et al.*, 2006. |
| Leopard frogs | *R. pipiens* | 2000 | Larvae | Atrazine | 20 | 200 | - | No significant effect on development rate, percent metamorphosis, time to metamorphosis, survival, mass at metamorphosis. | Allran & Karasov, 2000. |
| South American toad | *Rhinella arenarum* | 2008 | Embryos | atrazine | 1 | 5000 | - | No observed effects at lower concentrations (metamorphisis), large doses caused delayed motamorphisis and death (>5000 g $L^{-1}$) | Brodeur *et al.*. 2008 |
| Sprague-Dawley rats | | 2003 | Female | Atrazine | 50000 | 5000000 | 350 | No effect on induced rat ovarian carcinogenesis | Tanaka *et al.*, 2004 |
| Zebra fish | *Danio rerio* | 2003 | Juveniles | Atrazine | 1 | 100 nM | - | No influence of the conversion of androgens into estrogens | Kazeto *et al.*, 2004 |

| Common name | Sub species | Year | Age | Triazine | Minimum Exposure µg $L^{-1}$ | Maximum Exposure µg $L^{-1}$ | Experiment timeframe (days) | Observed effects | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **Test species with a significant observed affects from pesticide exposure** | | | | | | | | | |
| African Clawed Frog | *X. laevis* | 2002 | Juvenile | Atrazine | 0.1 | 400 | - | Development of testicular oocytes in male and ovotestis. | Hecker *et al.*, 2006. |
| Alligator | *Alligator mississippiensis* | 1996 | Eggs and juveniles | Atrazine | 140 | 140000 | Lab/ environment | Steroid hormone concentrations and gonadal-adrenal mesonephros aromatase activity reduced | Crain, 1997. |
| American Toads | *Bufo americanus* | 2004 | - | Atrazine | 3 | 100 | 30 | Survival was significantly lower for all animals exposed to 3 g $L^{-1}$ compared with either 30 or 100 g $L^{-1}$ | Storrs & Kiesecker, 2004a. |
| Amphibian tadpole | *Xenopus laevis* | 2001 | Tadpoles | Atrazine | - | 21 | 2 | Significantly reduce reproduction during the reproductive life of these animals | Tavera-Mendoza *et al.*, 2002. |
| Atlantic Salmon | *Salmo salar L.* | 1998 | Male Adults | Simazine | 0.1 | 2 | - | Reduced testosterone | Moore & Lower, 2001 |
| Atlantic Salmon | *Salmo salar L.* | 1998 | Male Adults | Atrazine | 0.5 | 2 | - | Reduced testosterone | Moore & Lower, 2001 |
| Atlantic Salmon | *Salmo salar L.* | 2007 | Juvenile | Atrazine | 10 | 100 | 21 | 100 ug $L^{-1}$ Atrazine exposure resulted in 9% mortality, irregularity, growth and endocrine disturbance. | Nieves-Puigdoller *et al.*, 2007. |
| Domestic Mouse | *Mus musculus* | 2005 | Gestational mice | Atrazine | 1 | 100 | 7 | Induced relevant neuronal damage in extra hypothalamic sites | Giusi *et al.*, 2006 |

| Common name | Sub species | Year | Age | Triazine | Minimum Exposure µg $L^{-1}$ | Maximum Exposure µg $L^{-1}$ | Experiment timeframe (days) | Observed effects | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Gold fish | *Carassius auratus L.* | 2006 | Adult | Atrazine, Simazine and others | - | 50 | 84 | Immune suppression | Fatima *et al.*, 2007 |
| Green Frogs | *Rana clamitans* | 2004 | - | Atrazine | 3 | 100 | 30 | Survival was significantly lower for all animals exposed to 3 µg $L^{-1}$ compared with either 30 or 100 µg $L^{-1}$ | Storrs & Kiesecker, 2004b |
| Green sea Turtle | *Chelonia mydas* | 2004 | Enzymes | Atrazine | 0.1 | 30 uM | - | Significantly induced aromatase activity (converts testosterone into estradiol) | Keller & McClellan-Green, 2004 |
| Japanese Quail eggs | *Coturnix coturnix japonica* | 2007 | Egg | Atrazine | 0.5 | 5 | - | Impaired reproduction | Ottinger *et al.*, 2008 |
| Leopard frogs | *R. pipiens* | 2003 | Juvenile | Atrazine | 0.1 | 400 | - | Development of testicular oocytes in male, ovotestis and decrease testicular development | Hayes *et al.*, 2003; Hecker *et al.*, 2006 |
| Leopard frogs | *R. pipiens* | 2007 | - | Atrazine | - | 21 | 8 | Immune suppression | Brodkin *et al.*, 2007 |
| Leopard frogs | *R. pipiens* | 2007 | - | Atrazine | - | >1 | Environmental survey | Atrazine exposed frogs had a high incidence of oocytes. Neither gonad size, gonad maturity nor sex steroid levels differed between normal males and those with testicular oocytes | Brodkin et al., 2007 |

| Common name | Sub species | Year | Age | Triazine | Minimum Exposure µg $L^{-1}$ | Maximum Exposure µg $L^{-1}$ | Experiment timeframe (days) | Observed effects | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Long-Evans hooded rats | - | 1999 | Female Adolescent | Atrazine | 51 mg/Kg | 301 mg/Kg | 21 | Suppressed estrogen | Cooper *et al.*, 2000 |
| Rat | *Rattus norvegicus* | 2002 | Juvenile male | - | - | 50 mg/kg | 12 | Testosterone levels reduced by 50% | Friedmann, 2002 |
| Sprague-Dawley rats | | 1999 | Female Adolescent | Atrazine | 50 mg/Kg | 300 mg/Kg | 21 | Suppressed estrogen | Cooper *et al.*, 2000 |
| Spring peepers frog | *Pseudacris crucifer* | 2004 | | Atrazine | 3 | 100 | 30 | Survival was significantly lower for all animals exposed to 3 g $L^{-1}$ compared with either 30 or 100 g $L^{-1}$ | Storrs & Kiesecker, 2004. |
| Swedish Landrace pigs | *Sus domestica* | 1999 | Female | Atrazine | - | 1 mg/Kg | 19 | Reduced estrogen during ovulation - in ability to reproduce as a result | Gojmerac et al., 1999. |
| Wistar rats | *Rattus norvegicus* | 1999 | juvenile | Atrazine | 12.5 mg/kg | 200 mg/kg | 30 | Delayed puberty, mortality above 50 mg/Kg. | In Stoker *et al.*, 2010 |
| Wood Frogs | *Rana sylvatica* | 2004 | | Atrazine | 3 | 100 | 30 | Survival was significantly lower for all animals exposed to 3 g $L^{-1}$ compared with either 30 or 100 g $L^{-1}$ | Storrs & Kiesecker, 2004 |
| Yorkshire Pigs | *Sus domestica* | 1999 | Female | Atrazine | - | 2 mg/Kg | 19 | Reduced estrogen during ovulation - in ability to reproduce as a result | Gojmerac *et al.*, 1999 |

# APPENDIX C: ATRAZINE CALIBRATION DATA

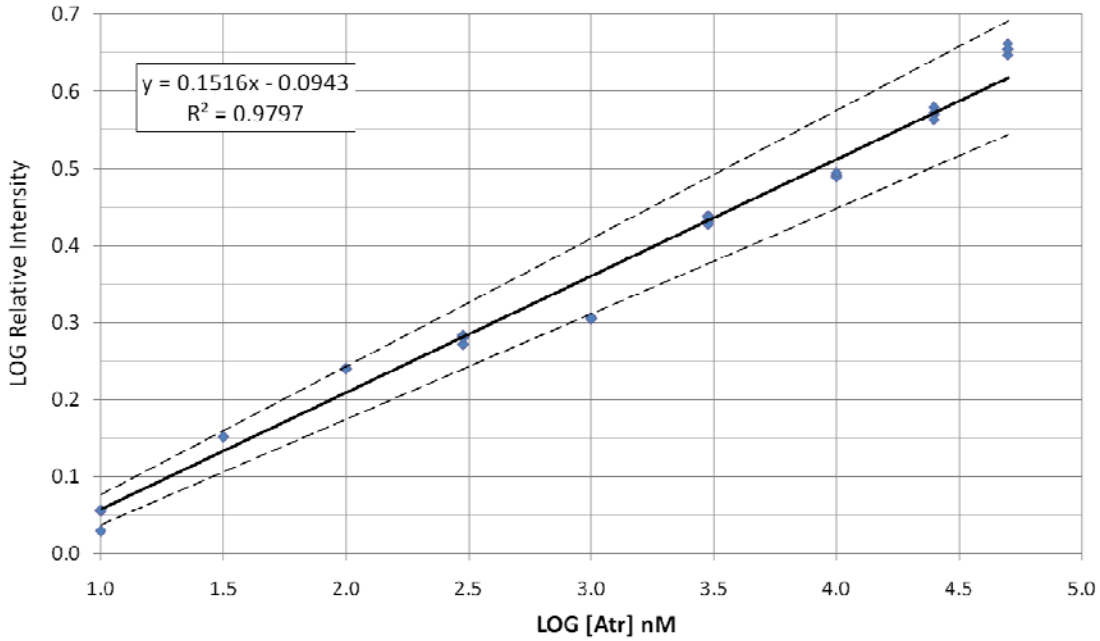



**Figure C-1: Atrazine calibration graph.**

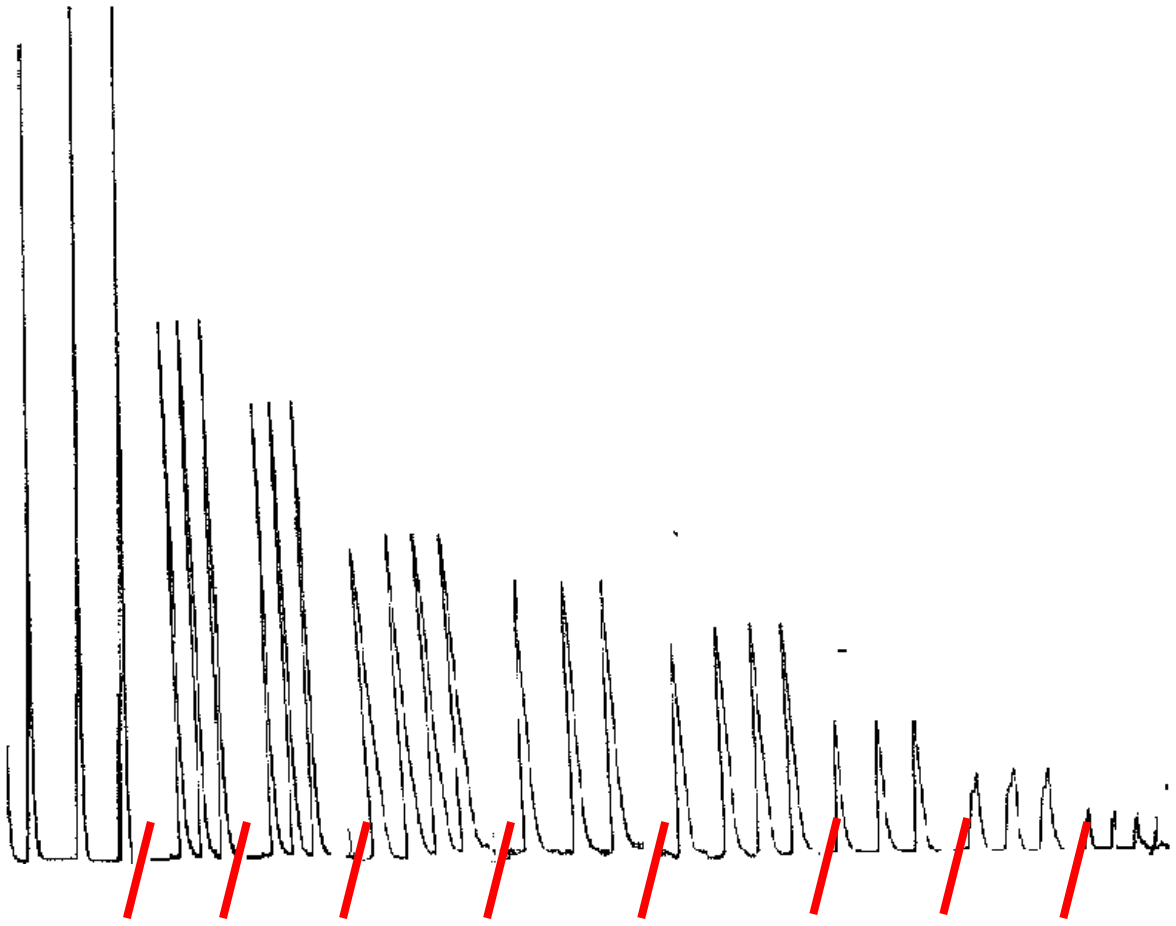


**Figure C-2: Atrazine standards in MilliQ water (Scanned and repositioned)**

# APPENDIX D: HPLC DEVELOPMENT TRIANGLES

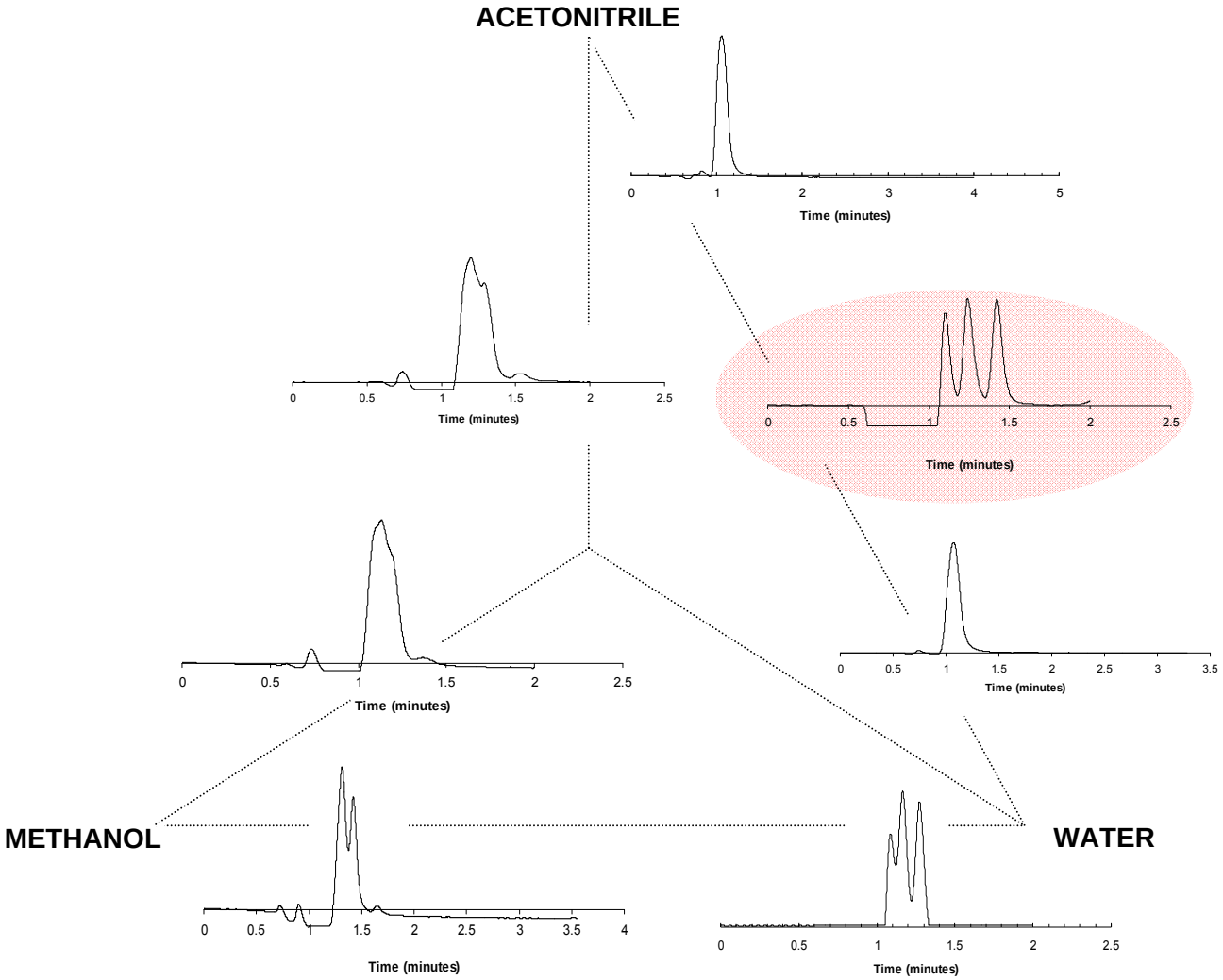



**Figure D-1: Application of the method development triangle for the separation of three atrazine metabolites by HPLC UV detection with a monolithic c8 reverse phase separation column.**

# APPENDIX E: ATR-FTIR ANALYSIS OF NATURAL WATERS

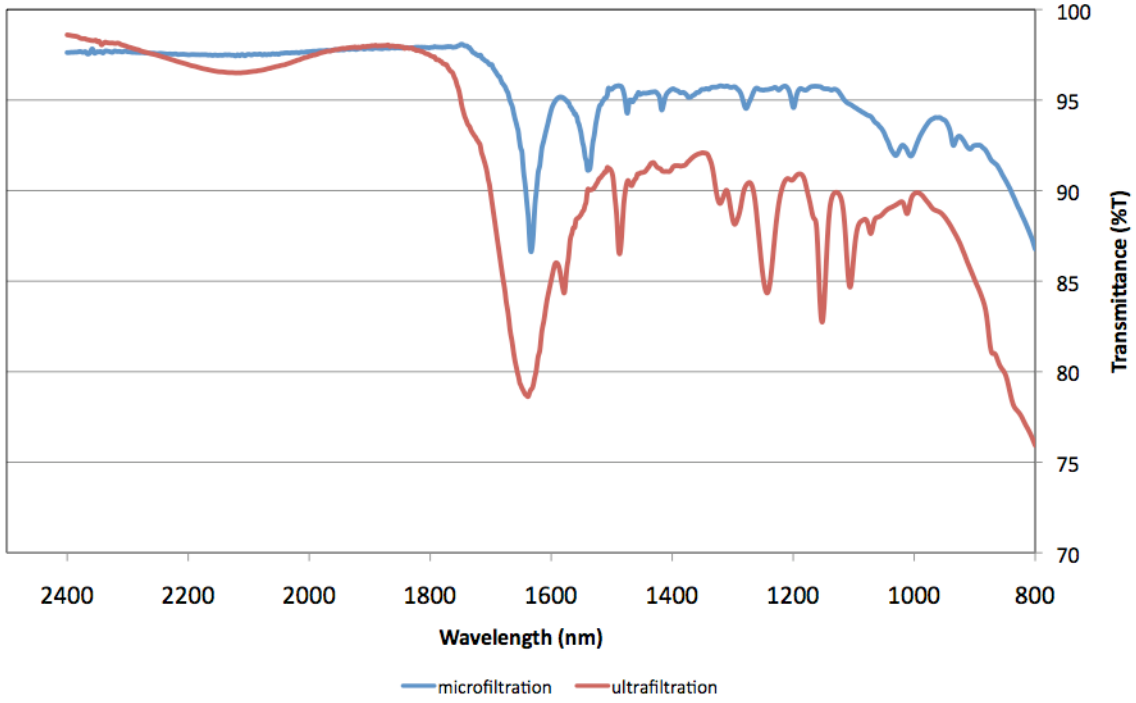



**Figure E-1: Natural water sample number one.**

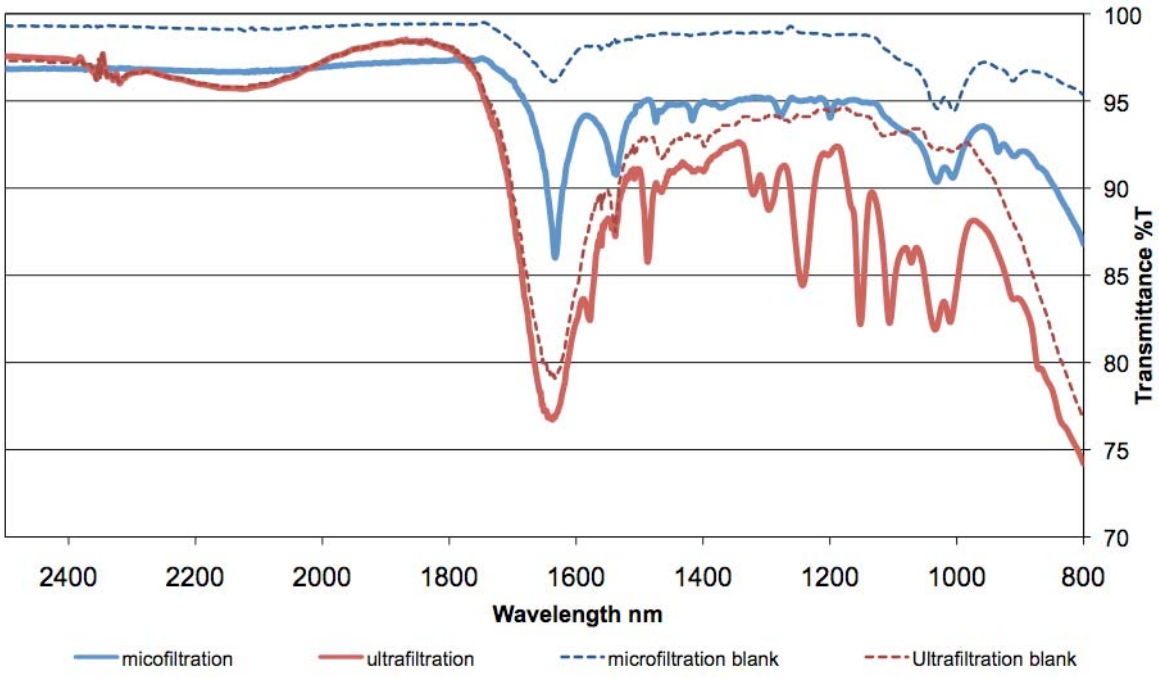



**Figure E-2: Natural water sample number two.**

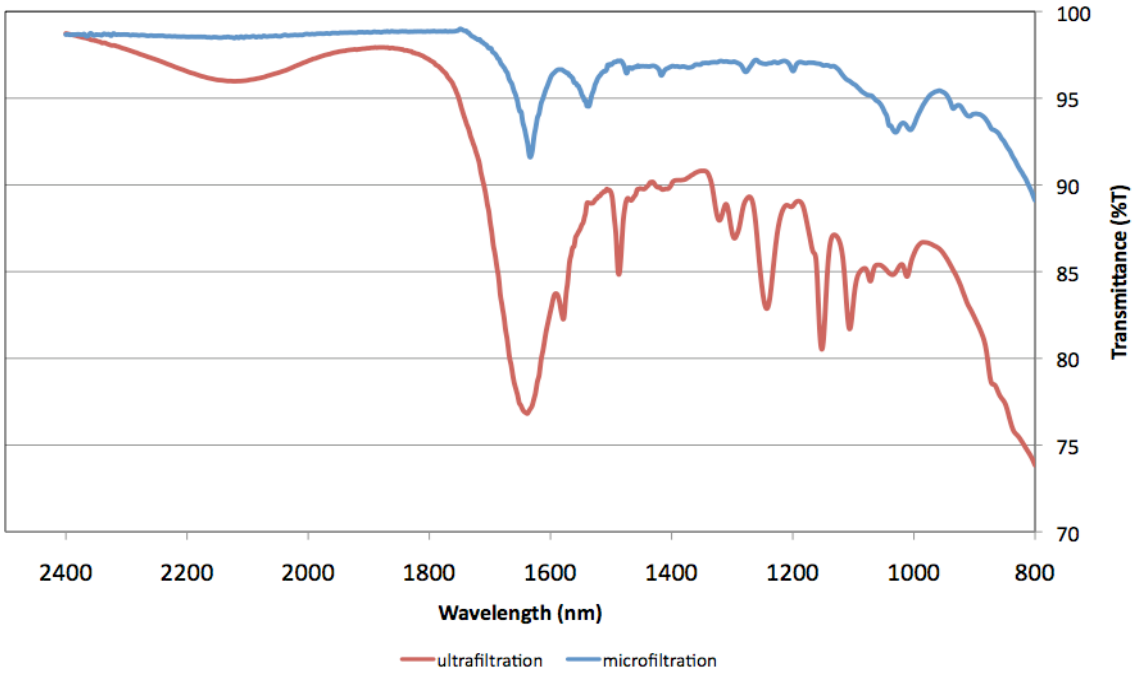



**Figure E-3: Natural water sample number three.**

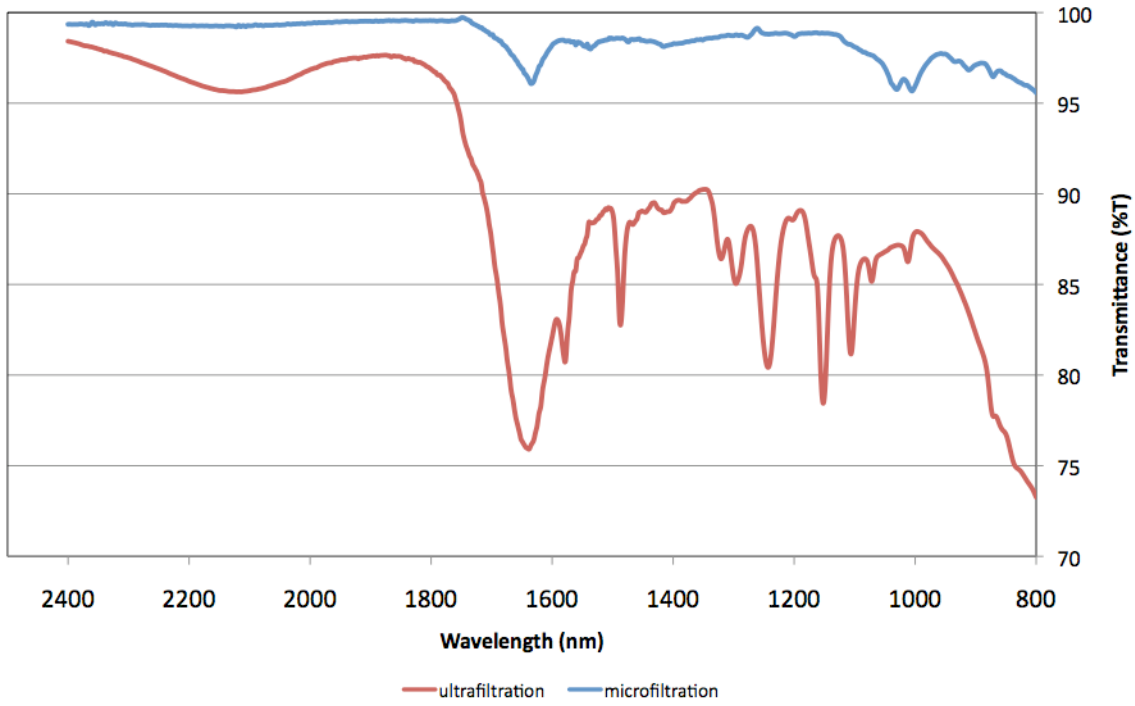



**Figure E-4: Natural water sample number four.**

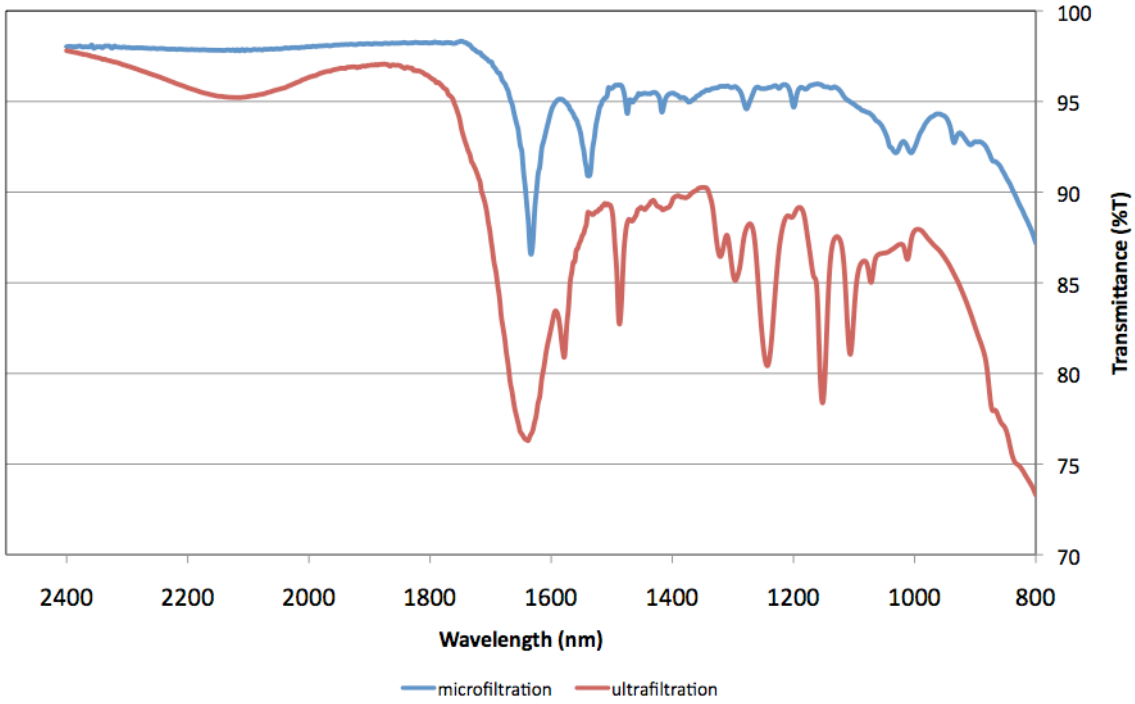



**Figure E-5: Natural water sample number five.**

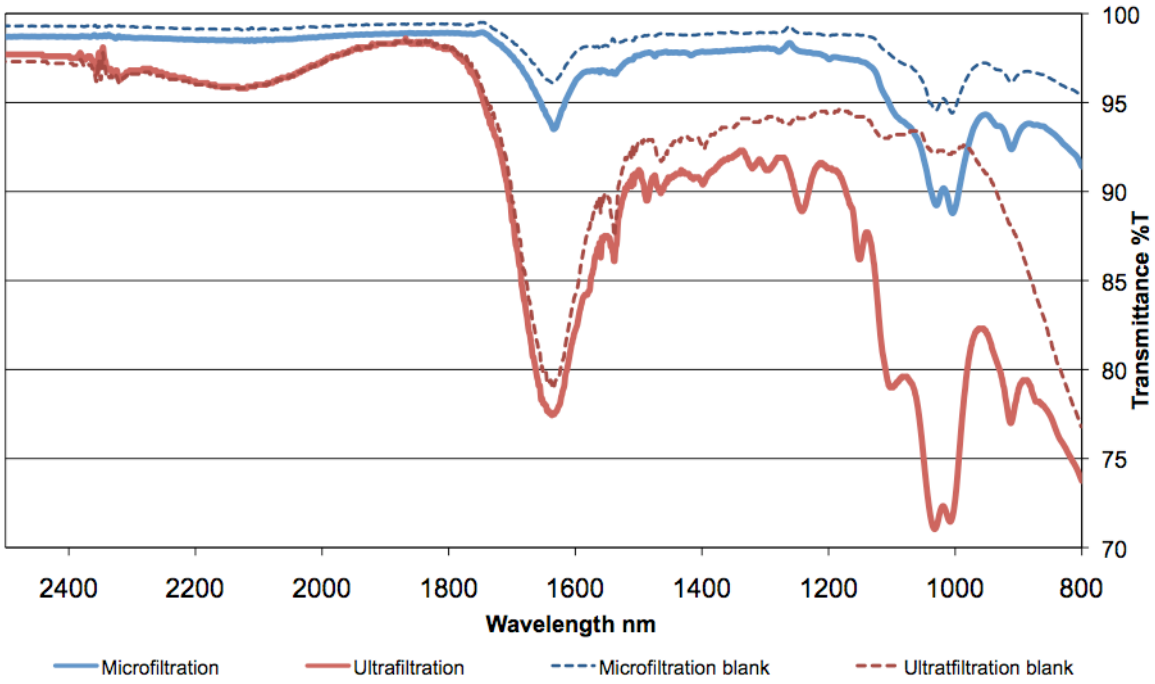



**Figure E-6: Natural water sample number six.**

# APPENDIX F: LUMINOL OPTIMISATION DATA

| Run# | Seq. # | Centre Point | Block # | [luminol] | [$H_2O_2$] | pH buffer | Flow rate | SBR |
|---|---|---|---|---|---|---|---|---|
| 7 | 1 | 1 | 1 | 0.5 | 0.5 | 10 | 0.5 | 1.8 |
| 2 | 2 | 1 | 1 | 5 | 0.2 | 2 | 0.5 | 1.9 |
| 20 | 3 | 0 | 1 | 2.75 | 0.35 | 7 | 2.25 | 6.4 |
| 11 | 4 | 1 | 1 | 0.5 | 0.5 | 2 | 4 | 0 |
| 10 | 5 | 1 | 1 | 5 | 0.2 | 2 | 4 | 1.7 |
| 4 | 6 | 1 | 1 | 5 | 0.5 | 2 | 0.5 | 1.7 |
| 16 | 7 | 1 | 1 | 5 | 0.5 | 10 | 4 | 2.0 |
| 19 | 8 | 0 | 1 | 2.75 | 0.35 | 7 | 2.25 | 6.4 |
| 9 | 9 | 1 | 1 | 0.5 | 0.2 | 2 | 4 | 0 |
| 5 | 10 | 1 | 1 | 0.5 | 0.2 | 10 | 0.5 | 1.9 |
| 13 | 11 | 1 | 1 | 0.5 | 0.2 | 10 | 4 | 2.0 |
| 14 | 12 | 1 | 1 | 5 | 0.2 | 10 | 4 | 0 |
| 6 | 13 | 1 | 1 | 5 | 0.2 | 10 | 0.5 | 2.0 |
| 18 | 14 | 0 | 1 | 2.75 | 0.35 | 7 | 2.25 | 3.3 |
| 12 | 15 | 1 | 1 | 5 | 0.5 | 2 | 4 | 2.7 |
| 15 | 16 | 1 | 1 | 0.5 | 0.5 | 10 | 4 | 2 |
| 3 | 17 | 1 | 1 | 0.5 | 0.5 | 2 | 0.5 | 0 |
| 17 | 18 | 0 | 1 | 2.75 | 0.35 | 7 | 2.25 | 12.7 |
| 1 | 19 | 1 | 1 | 0.5 | 0.2 | 2 | 0.5 | 0 |
| 8 | 20 | 1 | 1 | 5 | 0.5 | 10 | 0.5 | 2.4 |
| 27 | 1 | 1 | 2 | 0.5 | 0.5 | 10 | 0.5 | 2.0 |
| 22 | 2 | 1 | 2 | 5 | 0.2 | 2 | 0.5 | 2.2 |
| 40 | 3 | 0 | 2 | 2.75 | 0.35 | 7 | 2.25 | 6.8 |
| 31 | 4 | 1 | 2 | 0.5 | 0.5 | 2 | 4 | 0 |
| 30 | 5 | 1 | 2 | 5 | 0.2 | 2 | 4 | 1.9 |
| 24 | 6 | 1 | 2 | 5 | 0.5 | 2 | 0.5 | 1.6 |
| 36 | 7 | 1 | 2 | 5 | 0.5 | 10 | 4 | 2.2 |
| 39 | 8 | 0 | 2 | 2.75 | 0.35 | 7 | 2.25 | 6.8 |
| 29 | 9 | 1 | 2 | 0.5 | 0.2 | 2 | 4 | 0 |
| 25 | 10 | 1 | 2 | 0.5 | 0.2 | 10 | 0.5 | 2.0 |
| 33 | 11 | 1 | 2 | 0.5 | 0.2 | 10 | 4 | 2.1 |
| 34 | 12 | 1 | 2 | 5 | 0.2 | 10 | 4 | 0 |
| 26 | 13 | 1 | 2 | 5 | 0.2 | 10 | 0.5 | 2.0 |
| 38 | 14 | 0 | 2 | 2.75 | 0.35 | 7 | 2.25 | 3.5 |
| 32 | 15 | 1 | 2 | 5 | 0.5 | 2 | 4 | 2.9 |
| 35 | 16 | 1 | 2 | 0.5 | 0.5 | 10 | 4 | 2.1 |
| 23 | 17 | 1 | 2 | 0.5 | 0.5 | 2 | 0.5 | 0 |
| 37 | 18 | 0 | 2 | 2.75 | 0.35 | 7 | 2.25 | 13.7 |
| 21 | 19 | 1 | 2 | 0.5 | 0.2 | 2 | 0.5 | 0 |
| 28 | 20 | 1 | 2 | 5 | 0.5 | 10 | 0.5 | 2.6 |

# REFERENCES


Abaza, H. 1999. Environmental impacts of trade liberalization and policies for sustainable management of natural resources: A case study on Bangladesh's shrimp farming industry. *Trade Liberalization Environ.*

Aboul-Enein, H. Y., Stefan, R.-I., Van Staden, J. F., Zhang, X. R., Garcia-Campana, A. M. & Baeyens, W. R. G. 2000. Recent Developments and Applications of Chemiluminescence Sensors. *Critical Reviews in Analytical Chemistry,* 30, 271-289.

Abraxis. 2008. *Abraxis immunoassay method* [Online]. Available: http://www.abraxiskits.com/product_pesticides.htm [Accessed 2008 12/12].

Adcock, J. L., Barnett, N. W., Gerardi, R. D., Lenehan, C. E. & Lewis, S. W. 2004. Determination of glyphosate mono-isopropylamine salt in process samples using flow injection analysis with tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence detection. *Talanta,* 64, 534-537.

Adcock, J. L., Francis, P. S., Agg, K. M., Marshall, G. D. & Barnett, N. W. 2007. A hybrid FIA/HPLC system incorporating monolithic column chromatography. *Analytica Chimica Acta,* 600, 136-141.

Allran, J. W. & Karasov, W. H. 2000. EFFECTS OF ATRAZINE AND NITRATE ON NORTHERN LEOPARD FROG (RANA PIPIENS) LARVAE EXPOSED IN THE LABORATORY FROM POSTHATCH THROUGH METAMORPHOSIS. *Environmental Toxicology and Chemistry,* 19, 2850-2855.

Amis, A. 2008. *Friends of the Earth Melbourne and National Toxics Network* [Online]. Available: http://www.foe.org.au/resources/publications/general-environemntal-issues/Pesticide%20Report%20May%202008.pdf [Accessed November 2008].

AP&VMA (Australian Pesticides and Veterinary Medicines Authority). 2011. *Monocrotophos Review* [Online]. Available: http://www.apvma.gov.au/products/review/completed/monocrotophos.php [Accessed 1/11 2011].

Arnold, D. J., Bennett, D., Briggs, G. G., Cohen, S. Z., Huckle, K. R., Jones, R. L., Mikami, N., Millburn, P., Miyamoto, J., Parlar, H., Troberst, T. R., Smith, C. J. & Takimoto, Y. 1990. *Environmental Fate of Pesticides*, Wiley-Interscience.

Ayyagari, M. S., Kamtekar, S., Pande, R., Marx, K. A., Kumar, J., Tripathy, S. K., Akkara, J. & Kaplan, D. L. 1995. Chemiluminescence-based inhibition kinetics of alkaline phosphatase in the development of a pesticide biosensor. *Biotechnology progress,* 11, 699-703.

Baker, A. & Curry, M. 2004. Fluorescence of leachates from three contrasting landfills. *Water Research,* 38, 2605-2613.

Baker, A., Elliott, S. & Lead, J. R. 2007. Effects of filtration and pH perturbation on freshwater organic matter fluorescence. *Chemosphere,* 67, 2035-2043.

Baker, A. & Spencer, R. G. M. 2004. Characterization of dissolved organic matter from source to sea using fluorescence and absorbance spectroscopy. *Science of the Total Environment,* 333, 217-232.

Baranowska, I., Barchanska, H. & Pacak, E. 2006. Procedures of trophic chain samples preparation for determination of triazines by HPLC and metals by ICP-AES methods. *Environmental Pollution,* 143, 206-211.

Barnes, C. & Holz, G. Year. Environmental fate of atrazine applied pre- and post-planting in a Eucalyptus Nitens plantation in Tasmania, Australia. *In:* Weed Managment into the 21st Centruty - Do we know where we're going?, 1999. Australian Weed conference, 411-417.

Barnett, N. W., Lenehan, C. E. & Lewis, S. W. 1999. Sequential injection analysis: an alternative approach to process analytical chemistry. *TrAC Trends in Analytical Chemistry,* 18, 346-353.

Barzen, C., Brecht, A. & Gauglitz, G. 2002. Optical multiple-analyte immunosensor for water pollution control. *Biosensors and Bioelectronics,* 17, 289-295.

Bately, G., Crawford, C., Moore, M., Mcneil, J., Reid, J., Koehnken, L. & Ramsay, J. 2010. Report of the George River water Quality Panel: 'What's in the Water?". *In:* Panel, G. R. W. Q. (ed.). Tasmanian Government.

Baumner, A. J. & Schmid, R. D. 1998. Development of a new immunosensor for pesticide detection: a disposable system with liposome-enhancement and amperometric detection. *Biosensors and Bioelectronics,* 13, 519-529.

Beale, D. J., Dunn, M. S. & Marney, D. 2010. Application of GC-MS metabolic profiling to `blue-green water' from microbial influenced corrosion in copper pipes. *Corrosion Science,* 52, 3140-3145.

Bellomarino, S. A., Brown, A. J., Conlan, X. A. & Barnett, N. W. 2009. Preliminary evaluation of monolithic column high-performance liquid chromatography with tris(2,2'-bipyridyl)ruthenium(II) chemiluminescence detection for the determination of quetiapine in human body fluids. *Talanta,* 77, 1873-1876.

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. D. & Snyder, S. A. 2009. Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. *Environmental Science & Technology,* 43(3):597-603.

Bjarnason, B., Bousios, N., Eremin, S. & Johansson, G. 1997. Flow injection enzyme immunoassay of atrazine herbicide in water. *Analytica Chimica Acta,* 347, 111-120.

Bolden, M. E. & Danielson, N. D. 1998. Liquid chromatography of aromatic amines with photochemical derivatization and tris(bipyridine)ruthenium(III) chemiluminescence detection. *Journal of Chromatography A,* 828, 421-430.

Borman, C., Sullivan, B. P., Eggleston, C. & Colberg, P. 2009. The Use of Flow-Injection Analysis with Chemiluminescence Detection of Aqueous Ferrous Iron in Waters Containing High Concentrations of Organic Compounds. *Sensors,* 9, 4390-4406.

Box, G. E., Hunter, J. S. & Hunter, W. G. 2005. *Statistics for Experimenters: Design, Innovation, and Discovery*, Wiley.

Buchanan, W. 2008. *Personal communication*, Barwon Water, Australia.

Brodeur, J. C., Svartz, G., Perez-Coll, C. S., Marino, D. J. G. & Herkovits, J. 2009. Comparative susceptibility to atrazine of three developmental stages of Rhinella arenarum and influence on metamorphosis: Non-monotonous acceleration of the time to climax and delayed tail resorption. *Aquatic Toxicology,* 91(2):161-70.

Brodkin, M. A., Madhoun, H., Rameswaran, M. & Vatnick, I. 2007. Atrazine is an imune disruptor in adult northern leopard frogs (Rana Pipiens). *Environmental Toxicology and Chemistry,* 26, 80-84.

Broomhead, J. A. & Young, C. G. 1990. Tris(2,2'-bipyridine)ruthenium(II) dichloride hexahydrate. *Inorganic Syntheses,* 28, 334-340.

Brown, A. 2005. Mode of Action of Insecticides and Related Pest Control Chemicals for production agriculture, ornamentals, and turf. *Maryland Cooperative Extension.* University of Maryland.

Chantiwas, R. 2001. Combination of field-flow fractionation and flow injection analysis. *The Japan Society for Analytical Chemistry,* 17, 423-424.

Chen, J., Gu, B. H., Leboeuf, E. J., Pan, H. J. & Dai, S. 2002. Spectroscopic characterization of the structural and functional properties of natural organic matter fractions. *Chemosphere,* 48, 59-68.

Chen, J., Leboef, E. J., Dai, S. & Gu, B. H. 2003. Fluorescence spectroscopic studies of natural organic matter fractions. *Chemosphere,* 50, 639-647.

Chocholous, P., Satínský, D., Sladkovský, R., Pospísilová, M. & Solich, P. 2008. Determination of pesticides fenoxycarb and permethrin by sequential injection chromatography using miniaturized monolithic column. *Talanta,* 77, 566-570.

Chow, C., Fabris, R., Wilkinson, K., Fitzgerald, F. & Drikas, M. 2006. Characterisng NOM to assess treatability. *Water,* March.

Ciumasu, I. M., Kramer, P. M., Weber, C. M., Kolb, G., Tiemann, D., Windisch, S., Frese, I. & Kettrup, A. A. 2005. A new, versatile field immunosensor for environmental pollutants: Development and proof of principle with TNT, diuron, and atrazine. *Biosensors and Bioelectronics,* 21, 354-364.

Coady, K. K., Murphy, M. B., Villeneuve, D. L., Hecker, M., Jones, P. D., Carr, J. A., Solomon, K. R., Smith, E. E., Van Der Kraak, G., Kendall, R. J. & Giesy, J. P. 2005. Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in Xenopus laevis. *Ecotoxicology and Environmental Safety,* 62, 160-173.

Coly, A. & Aaron, J. 1999. Sensitive and flow injection analysis of sulfonyurea herbicides in water with micellar-enchanced photochemically induced fluroescence detection. *Analytica Chimica Acta,* 392, 255-264.

Considine, R. 2005. *Personal communication*. Melbourne Water, Australia.

Cooper, R. L. 2008. *Research addresses the potential risks of strazine and related pesticides* [Online]. EPA. Available: http://www.epa.gov/hhrp/factsheets/potential-risks-of-atrazine-and-related-pesticides.html [Accessed 10/12 2008].

Cooper, R. L., Stoker, T. E., Tyrey, L., Goldman, J. M. & Mcelroy, W. K. 2000. Atrazine Disrupts the Hypothalamic Control of Pituitary-Ovarian Function. *Toxicol. Sci.,* 53, 297-307.

Costin, J. W., Barnett, N. W. & Lewis, S. W. 2004a. Determination of proline in wine using flow injection analysis with tris(2,2'-bipyridyl)ruthenium(II) chemiluminescence detection. *Talanta,* 64, 894-898.

Costin, J. W., Barnett, N. W. & Lewis, S. W. 2004b. Determination of proline in wine using flow injection analysis with tris(2,2'-bipyridyl)ruthenium(II) chemiluminescence detection. *Talanta,* 64, 894-898.

Costin, J. W., Francis, P. S. & Lewis, S. W. 2003. Selective determination of amino acids using flow injection analysis coupled with chemiluminescence detection. *Analytica Chimica Acta,* 480, 67-77.

Crain, D. A. 1997. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. *Environmental Health Perspectives,* 105, 528-533.

Crow, J. M. 2011. Queensland floods hit Great Barrier Reef. *Nature News* [Online]. Available: http://www.nature.com/news/2011/110118/full/news.2011.21.html [Accessed 1/2].

Davies, P. E., Cook, L. S. J. & Barton, J. L. 1994. TRIAZINE HERBICIDE CONTAMINATION OF TASMANIAN STREAMS - SOURCES, CONCENTRATIONS AND EFFECTS ON BIOTA. *Australian Journal of Marine and Freshwater Research,* 45, 209-226.

Davis, G. 2004. *Tasmania: name your poison,* The Sunday Program (Channel 9). Accessed: 26/12/2004. Televsion. Produced Rushworth, D. Australia.

De La Cruz, A. M. D. A., Burguera, J. L., Burguera, M. & Rivas, C. 1995. Factorial design for the response exploration of a flow injection system. *Talanta,* 42, 701-709.

DECC&W (Department of Environment, Climate Change and Water). 2011. Water quality and river health report [Online]. Available: http://www.environment.nsw.gov.au/water/catchmentwq.htm [Accessed 17/2/2011].

DE&H (Department of the environment and Heritage, Australian Government). 2001. *State of the Environment report* [Online]. Available: http://www.deh.gov.au/soe/ [Accessed 1/1 2005].

DE&H (Department of the environment and Heritage, Australian Government). 2006. *State of the Environment report* [Online]. Available: http://www.deh.gov.au/soe/ [Accessed 1/1 2005].

Delgado Reyes, F., Fernández Romero, J. M. & Luque De Castro, M. D. 2000. Selective inhibition-based biosensing system for the determination of pesticides in environmental samples using analytical pervaporation coupled with enzymatic derivatisation. *Analytica Chimica Acta,* 408, 209-216.

Deluca, M., Fraser, M. E., Grayeski, M. L., Kenner, R. D., Lin, M. C., Lee, J., Mccapra, F., Mcelroy, W. D., Mohan, A. G., Orgryzlo, E. A., Perring, K. D., Roswell, D. F., Slawinska, D., Slawinski, J., Stedman, D. H., Umstead, M. E., Wampler, J. E., Ward, W. W. & White, E. H. 1985. *Chemi- and Bioluminescence*, Marcel Dekker.

Denholm, M. 2008. *Alarm at weed-kill chemical in water* [Online]. The Australian. Available: http://www.theaustralian.news.com.au/story/0,25197,23700909-30417,00.html [Accessed 10/1 2009].

Determann, S., Lobbes, J. M., Reuter, R. & Rullkötter, J. 1998. Ultraviolet fluorescence excitation and emission spectroscopy of marine algae and bacteria. *Marine Chemistry,* 62, 137-156.

Diaz, L., Llorca-Porcel, J. & Valor, I. 2008. Ultra trace determination of 31 pesticides in water samples by direct injection-rapid resolution liquid chromatography-electrospray tandem mass spectrometry. *Analytica Chimica Acta,* 624, 90-96.

Dixon, B. 2004. Pushing Bordeaux mixture. *The Lancet Infectous Diseases,* 4, 594-594.

Dousset, S., Chauvin, C., Durlet, P. & Thevenot, M. 2004. Transfer of hexazinone and glyphosate through undisturbed soil columns in soils under Christmas tree cultivation. *Chemosphere,* 57, 265-272.

Du, J., Liu, X. & Lu, J. 2003. Determination of Monocrotophos Pesticide by Flow Injection Chemiluminescence Method Using Luminol-Hydrogen Peroxide System. *Analytical Letters,* 36, 1029-1038.

Du Preez, L. H., Kunene, N., Everson, G. J., Carr, J. A., Giesy, J. P., Gross, T. S., Hosmer, A. J., Kendall, R. J., Smith, E. E., Solomon, K. R. & Van Der Kraak, G. J. 2008. Reproduction, larval growth, and reproductive development in African clawed frogs (Xenopus laevis) exposed to atrazine. *Chemosphere,* 71, 546-552.

Elliott, S., Lead, J. R. & Baker, A. 2006. Characterisation of the fluorescence from freshwater, planktonic bacteria. *Water Research,* 40, 2075-2083.

Emneus, J., Jain, S. R., Borowska, E., Davidsson, R., Tudorache, M. & Ponten, E. 2004. A chemiluminescence flow immunosensor based on a porous monolithic metacrylate and polyethylene composite disc modified with Protein G. *Biosens. Bioelectron.,* 19, 795-803.

Evgenidou, E. & Fytianos, K. 2002. Photodegradation of Triazine Herbicides in Aqueous Solutions and Natural Waters. *Journal of Agricultural and Food Chemistry,* 50, 6423-6427.

Extonet. 1995a. *Pesticide Information Profiles: Dicrotophos* [Online]. Oregon State University. Available: http://extoxnet.orst.edu/pips/dicrotop.htm [Accessed 2008].

Extonet. 1995b. *Pesticide Information Profiles: Monocrotophos* [Online]. Oregon State University. Available: http://extoxnet.orst.edu/pips/monocrot.htm [Accessed 2008].

Extonet. 1996a. *Pesticide Information Profiles: Atrazine* [Online]. Oregon State University. Available: http://extoxnet.orst.edu/pips/atrazine.htm [Accessed 2008].

Extonet. 1996b. *Pesticide Information Profiles: Hexazinone* [Online]. Oregon State University. Available: http://extoxnet.orst.edu/pips/hexazin.htm [Accessed 2008].

Extonet. 1996c. *Pesticide Information Profiles: Simazine* [Online]. Oregon State University. Available: http://extoxnet.orst.edu/pips/simazine.htm [Accessed 2008].

Fähnrich, K. A., Pravda, M. & Guilbault, G. G. 2001. Recent applications of electrogenerated chemiluminescence in chemical analysis. *Talanta,* 54, 531-559.

Farran, A., De Pablo, J. & Hernã¡Ndez, S. 1992. Continuous Flow Determination of Organophosphorus Pesticides Using Solid Phase Extraction Coupled On-Line with High Performance Liquid Chromatography. *International Journal of Environmental Analytical Chemistry,* 46, 245 - 253.

Farran, A., De Pablo, J. & Hernández, S. 1988. Continuous-flow extraction of organophosphorus pesticides coupled on-line with high-performance liquid chromatography. *Analytica Chimica Acta,* 212, 123-131.

Farre, M., Martinez, E., Ramin, J., Navarro, A., Radjenovic, J., Mauriz, E., Lechuga, L., Marco, M. & Barcel, D. 2007. Part per trillion determination of atrazine in natural water samples by a surface plasmon resonance immunosensor. Analytical and Bioanalytical Chemistry, 388, 207-214.

Fatima, M., Mandiki, S. N. M., Douxfils, J., Silvestre, F., Coppe, P. & Kestemont, P. 2007. Combined effects of herbicides on biomarkers reflecting immune-endocrine interactions in goldfish. *Aquatic Toxicology,* 81, 159-167.

Ferré, J., Bogue, R., Fernandez-Band, B., Larrechi, M. S. & Rius, F. X. 1997. Figures of merit in multivariate calibration. Determination of four pesticides in water by flow injection analysis and spectrometric detection. *Analtica Chimca Acta,* 348, 167-175.

Fishel, F. 2006. Pesticides and the Environment. *In:* Ofmissouri-Columbia, U. (ed.) *Agriculture MU Guide.*

Fránek, M., Deng, A. & Kolár, V. 2000. Performance characteristics for flow injection immunoassay using monoclonal antibodies against s-triazine and 2,4-D herbicides. *Analytica Chimica Acta,* 412, 19-27.

Franzmann, P. D., Zappia, L. R., Patterson, B. M. & Rayner, J. L. 1998. Mineralisation of low concnetrations of organic compounds and microbial biomass in surface and vadose zone soils from the swan caoastal plain, Western Australiawaters. *Australian Journal of Soil Research,* 36, 921-939.

Friedmann, A. S. 2002. Atrazine inhibition of testosterone production in rat males following peripubertal exposure. *Reproductive Toxicology,* 16, 275-279.

Fry, M., Fenwick, R. & Zinn, N. 2001. *American Brid Consevancy - Pesticides and Birds Compaign* [Online]. US EPA. Available: http://www.epa.gov/pesp/member_pages/abcpbc.htm [Accessed 12/12 2008].

Fu, P. Q., Wu, F. C., Liu, C. Q., Wang, F. Y., Li, W., Yue, L. X. & Guo, Q. J. 2007. Fluorescence characterization of dissolved organic matter in an urban river and its complexation with Hg(II). *Applied Geochemistry,* 22, 1668-1679.

Galeano Diaz, T., Acedo Valenzuela, M. I. & Salinas, F. 1999. Determination of the pesticide Naptalam, at the ppb level, by FIA with fluorimetric detection and on-line preconcentration by solid-phase extraction on C18 modified silica. *Analytica Chimica Acta,* 384, 185-191.

Gamiz-Gracia, L., García-Campana, A. M., Soto-Chinchilla, J. J., Huertas-Perez, J. F. & Gonzalez-Casado, A. 2005. Analysis of pesticides by chemiluminescence detection in the liquid phase. *TrAC Trends in Analytical Chemistry,* 24, 927-942.

García Reyes, J. F., Ortega Barrales, P. & Molina Díaz, A. 2003. Gel-surface fluorescence sensing system coupled with a continous-flow assembly for simultaneous monitoring of benomyl and carbendazim. *Analytica Chimica Acta,* 493, 34-45.

Gascón, J., Oubiña, A., Ballesteros, B., Barceló, D., Camps, F., Marco, M.-P., González-Martínez, M. A., Morais, S., Puchades, R. & Maquieira, A. 1997. Development of a highly sensitive enzyme-linked immunosorbent assay for atrazine Performance evaluation by flow injection immunoassay. *Analytica Chimica Acta,* 347, 149-162.

Geerdink, R. B., Niessen, W. M. A. & Brinkman, U. A. T. 2001. Mass spectrometric confirmation criterion for product-ion spectra generated in flow-injection analysis: Environmental application. *Journal of Chromatography A,* 910, 291-300.

Gerardi, R. D., Barnett, N. W. & Jones, P. 1999. Two chemical approaches for the production of stable solutions of tris(2,2'-bipyridyl)ruthenium(III) for analytical chemiluminescence. *Analytica Chimica Acta,* 388, 1-10.

Gervais, G., Brosillon, S., Laplanche, A. & Helen, C. 2008. Ultra-pressure liquid chromatography-electrospray tandem mass spectrometry for multiresidue determination of pesticides in water. *Journal of Chromatography A,* 1202, 163-172.

Giusi, G., Facciolo, R. M., Canonaco, M., Alleva, E., Belloni, V., Dessi'-Fulgheri, F. & Santucci, D. 2006. The Endocrine Disruptor Atrazine Accounts for a Dimorphic Somatostatinergic Neuronal Expression Pattern in Mice. *Toxicol. Sci.,* 89, 257-264.

Gojmerac, T., Uremoviæ, M., Uremoviæ, Z., Uriæ, S. & Bilandiæ, N. 1999. Reproductive disturbance caused by an S-triazine herbacide in pigs. *Acta Veterinaria Hungarica,* 47, 129-135.

Gok, E. & Ates, S. 2004. Determination of thyroxine hormone by luminol chemiluminescence. *Analytica Chimica Acta,* 505, 125-127.

Gorman, B. A., Francis, P. S. & Barnett, N. W. 2006. Tris(2,2'-bipyridyl)ruthenium (II) chemiluminescence. *The Analyst,* 131, 616-639.

Gorman, B. A., Lim, K. F., Hogan, C. F. & Barnett, N. W. 2007. Effect of oxidant type on the chemiluminescence intensity from the reaction of tris(2,2'-bipyridyl)ruthenium(III) with various organic acids. *Talanta,* 72, 568-574.

Graymore, M., Stagnitti, F. & Allinson, G. 2001. Impacts of atrazine in aquatic ecosystems. *Environmental International,* 26, 483-495.

Great Barrier Reef Marine Park authority, 2008. *Research Priorities* [Online]. Great Barrier Reef Marin Park Authority. Available: http://www.gbrmpa.gov.au/corp_site/key_issues/water_quality/research_priorities [Accessed 10/12 2008].

Gustafson, D. I. 1988. Groundwater Ubiquity Score: A simple method for assessing pesticide leachability. *Environmental Toxicology and Chemistry,* 8, 339-357.

Guzzella, L., Pozzoni, F. & Giuliano, G. 2006. Herbicide contamination of surficial groundwater in Northern Italy. *Environmental Pollution,* 142, 344-353.

Hamilton, D. J., Ambrus, A., Dieterle, R. M., Felsot, A. S., Harris, C. A., Holland, P. T., Katayama, A., Kurihara, N., Linders, J., Unsworth, J. & Wong, S. S. 2003. Regulatory limits for pesticide residues in water - (IUPAC Technical Report). *Pure and Applied Chemistry,* 75, 1123-1155.

Hansen, E. H., Ghose, A. K. & Ruzicka, J. 1977. Flow Injection analysis of Environmenatal Samples for Nitrite Using Ion-selective Electrode. *Analyst,* 102, 705-713.

Hassan, S. S. M., Abbas, M. N. & Moustafa, G. A. E. 1998. Flow Injection Potentiometric Determination of Atrazine in Herbicide Formulations. *Analytical Letters,* 31, 777-791.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C. & Vonk, A. 2002. Herbicides: Feminization of male frogs in the wild. *Nature,* 419, 895-896.

Hayes, T., Haston, K., Tsui, M., Hoang, A., Haeffele, C. & Vonk, A. 2003. Atrazine-Induced Hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and Field Evidence. *Environmental Health Perspectives,* 111, 568-575.

Haynes, D., Müller, J. & Carter, S. 2000. Pesticide and Herbicide Residues in Sediments and Seagrasses from the Great Barrier Reef World Heritage Area and Queensland Coast. *Marine Pollution Bulletin,* 41, 279-287.

Hecker, M., Murphy, M. B., Coady, K. K., Villeneuve, D. L., Jones, P. D., Carr, J. A., Solomon, K. R., Smith, E. E., Kraak, G., Gross, T., Preez, L., Kendall, R. J. & Giesy, J. P. 2006. Terminology of Gonadal Anomalies in Fish and Amphibians Resulting from Chemical Exposures. *Reviews of Environmental Contamination and Toxicology.*

Henderson, R. K., Baker, A., Murphy, K. R., Hambly, A., Stuetz, R. M. & Khan, S. J. 2009. Fluorescence as a potential monitoring tool for recycled water systems: A review. *Water Research,* 43, 863-881.

Holeman, J. A. & Danielson, N. D. 1994. Liquid chromatography of antihistamines using post-column tris(2,2'-bipyridine) ruthenium(III) chemiluminescence detection. *Journal of Chromatography A,* 679, 277-284.

Huang, L., Qiu, S., Pinzon, N. M., Lien, L. T. & Ju, L. K. 2008. Fluorescence characteristics of triazine-manufacturing wastewater. *Environmental Engineering Science,* 25, 439-449.

Hudson, N., Baker, A. & Reynolds, D. 2007. Fluorescence analysis of Dissolved Oganic Matter in natural, waste and polluted waters- A Review. *River Research and Applications,* 23, 631-649.

Huertas-Perez, J. F., Gamiz-Gracia, L., Garcia-Campana, A. M., Gonzalez-Casado, A. & Martinez Vidal, J. L. 2005. Chemiluminescence determination of carbofuran at trace levels in lettuce and waters by flow-injection analysis. *Talanta,* 65, 980-985.

Huertas-Perez, J. F., Garcia-Campana, A. M., Gamiz-Gracia, L., Gonzalez-Casado, A. & Iruela, M. D. O. 2004. Sensitive determination of carbaryl in vegetal food and natural waters by flow-injection analysis based on the luminol chemiluminescence reaction. *Analytica Chimica Acta,* 524, 161-166.

Icardo, M. C., Mateo, J. V. G., Lozano, M. F. & Calatayud, J. M. 2003. Enhanced flow-injection-chemiluminometric determination of sulphonamides by on-line photochemical reaction. *Analytica Chimica Acta,* 499, 57-69.

Jain, S. R., Borowska, E., Davidsson, R., Tudorache, M., Ponten, E. & Emneus, J. 2004. A chemiluminescence flow immunosensor based on a porous monolithic metacrylate and polyethylene composite disc modified with Protein G. *Biosensors and Bioelectronics,* 19, 795-803.

Jiang, F. H., Lee, F. S. C., Wang, X. R. & Dai, D. J. 2008. The application of Excitation/Emission Matrix spectroscopy combined with multivariate analysis for the characterization and source identification of dissolved organic matter in seawater of Bohai Sea, China. *Marine Chemistry,* 110, 109-119.

Jiang, T., Halsall, H. B., Heineman, W. R., Giersch, T. & Hock, B. 1995. Capillary Enzyme Immunoassay with Electrochemical Detection for the Determination of Atrazine in Water. *Journal of Agricultural and Food Chemistry,* 43, 1098-1104.

Jooste, A. M., Du Preez, L. H., Carr, J. A., Giesy, J. P., Gross, T. S., Kendall, R. J., Smith, E. E., Van Der Kraak, G. L. & Solomon, K. R. 2005. Gonadal Development of Larval Male Xenopus laevis Exposed to Atrazine in Outdoor Microcosms. *Environmental Science & Technology,* 39, 5255-5261.

Junkerbuchheit, A. & Witzenbacher, M. 1996. Pesticide monitoring of drinking water with the help of solid-phase extraction and high-performance liquid chromatography. *Journal of Chromatography A,* 737, 67-74.

Karlberg, B. & Pacey, G. E. 1998. *Flow Injection Analysis: a Practical Guide*, Elsevier Science Publishing.

Kazeto, Y., Place, A. R. & Trant, J. M. 2004. Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (Danio rerio) juveniles. *Aquatic Toxicology,* 69, 25-34.

Keller, J. M. & Mcclellan-Green, P. 2004. Effects of organochlorine compounds on cytochrome P450 aromatase activity in an immortal sea turtle cell line. *Marine Environmental Research,* 58, 347-351.

Klinkenberg, A. & Sjenitzer, F. 1956. Holding-time distributions of the Gaussian type. *Chemical Engineering Science,* 5, 258-270.

Knox, D. 2010. *Something in the Water*, Australian Story (ABC1). Accessed: 15/2/2010. Television.

Kodamatani, H., Shimizu, H., Saito, K., Yamazaki, S. & Tanaka, Y. 2006. High-performance liquid chromatography of aromatic compounds with photochemical decomposition and tris(2,2'-bipyridine)ruthenium(III) chemiluminescence detection. *Journal of Chromatography A,* 1102, 200-205.

Kookana, R. S., Baskaran, S. & Naidu, R. 1998. Pesticide fate and behaviour in Australian soils in relation to contamination and management of soil and water: a review. *Australian Journal of Soil Research,* 36, 715-764.

Kookana, R. S., Di, H. J. & Aylmore, A. G. 1995. A field study of leaching and degradation of nine pesticides in sandy soil. *Australian Journal of Soil Research,* 33, 1019-1030.

Korth, W. 1995. Can Pesticides used in irrigated agriculture get into drainage water. *Farmes Newsletter* [Online], 145.

Krämer, P. M., Baumann, B. A. & Stoks, P. G. 1997. Prototype of a newly developed immunochemical detection system for the determination of pesticide residues in water. *Analytica Chimica Acta,* 347, 187-198.

Krämer, P. M., Franke, A. & Standfuss-Gabisch, C. 1999. Flow injection immunoiffinity analysis (FIIAA): a screening technology for atrazine and diuron in water samples. *Analytica Chimica Acta,* 399, 199.

La Rosa, C., Pariente, F., Hernández, L. & Lorenzo, E. 1994. Determination of organophosphorus and carbamic pesticides with an acetylcholinesterase amperometric biosensor using 4-aminophenyl acetate as substrate. *Analytica Chimica Acta,* 295, 273-282.

La Rosa, C., Pariente, F., Hernández, L. & Lorenzo, E. 1995. Amperometric flow-through biosensor for the determination of pesticides. *Analytica Chimica Acta,* 308, 129-136.

Lane, D. A. & Sirs, J. A. 1974. Indicator dilution measurement of mean transit time and flow in a straight tube. *Journal of Physics E: Scientific Instruments,* 7, 51-55.

Law, K. A. & J. Higson, S. P. 2005. Sonochemically fabricated acetylcholinesterase micro-electrode arrays within a flow injection analyser for the determination of organophosphate pesticides. *Biosensors and Bioelectronics,* 20, 1914-1924.

Lee, H.-S., Ah Kim, Y., Ae Cho, Y. & Tae Lee, Y. 2002. Oxidation of organophosphorus pesticides for the sensitive detection by a cholinesterase-based biosensor. *Chemosphere,* 46, 571-576.

Lee, S., Kwon, B., Sun, M. & Cho, J. 2005. Characterizations of NOM included in NF and UF membrane permeates. *Desalination,* 173, 131-142.

Lee, W.-Y. & Nieman, T. A. 1996. Effect of organic solvent on tris(2,2'-bipyridyl)ruthenium(III) chemiluminescent reactions in flowing streams. *Analytica Chimica Acta,* 334, 183-191.

Levenspiel, O. 1958. Longitudinal Mixing of Fluids Flowing in Circular Pipes. *Industrial & Engineering Chemistry,* 50, 343-346.

Levenspiel, O. & Smith, W. K. 1957. Notes on the diffusion-type model for the longitudinal mixing of fluids in flow. *Chemical Engineering Science,* 6, 227-235.

Levenspiel, O. & Turner, J. C. R. 1970. The interpretation of residence-time experiments. *Chemical Engineering Science,* 25, 1605-1609.

Lewis, S. E., Brodie, J. E., Bainbridge, Z. T., Rohde, K. W., Davis, A. M., Masters, B. L., Maughan, M., Devlin, M. J., Mueller, J. F. & Schaffelke, B. 2009. Herbicides: A new threat to the Great Barrier Reef. *Environmental Pollution,* 157, 2470-2484.

Long, G. L. & Winefordner, J. D. 1983. *Analytical Chemistry,* 55, 713-724.

Kolev, S.D. & McKelvie, I.D. 2008. Advances in flow injection analysis and related techniques. *Comprehensive Analytical Chemistry.* Elsevier.

Lu, J. R. & Xiaoyu Liu, J. D. J. L. 2003. Determination of parathion residues in rice samples using a flow injection chemiluminescence method. *Luminescence,* 18, 245-248.

Luna, J. R., Ovalles, J. F., León, A. & Buchheister, M. 2000. A factorial design for optimizing a flow injection analysis system. *Fresenius' Journal of Analytical Chemistry,* 367, 201-203.

Mackenzie, D. 2008. *Switch to biofuels boosts insect pest* [Online]. New Scientist magazine. Available: http://www.newscientist.com/article/dn16279-switch-to-biofuels-boosts-insect-pest.html [Accessed 5/1 2009].

Mallat, E., Klotz, A., Brecht, A., Gauglitz, G. & Barcelo, D. 1999. River Analyzer for Chlorotriazines with a Direct Optical Immunosensor. *Environmental Science & Technology,* 33, 965-971.

Manciulea, A., Baker, A. & Lead, J. R. 2009. A fluorescence quenching study of the interaction of Suwannee River fulvic acid with iron oxide nanoparticles. *Chemosphere,* 76, 1023-1027.

Maniasso, N., Zagatto, E. A. G., Reis, S., Santos, J. L. M. & Lima, J. L. F. C. 1999. Exploitation of micellar medium for photochemical-spectrofluorimetric flow-injection determination of fenvalerate. *Laboratory Automation & Information Management,* 34, 143-148.

Mansour, M., Feicht, E. A., Behechti, A. & Scheurert, I. 1997. Experimental approaches to studying the photostability of selected pesticides in water and soil. *Chemosphere,* 35, 39.

Mansur, M. & Feicht, E. A. 1994. Transformation of chemical contaminants by biotic and abiotic processes in water and soil. *Chemosphere,* 28, 323.

Marquette, C. A. & Blum, L. J. 1998. Electrochemiluminescence of luminol for 2,4-D optical immunosensing in a flow injection analysis system. *Sensors and Actuators B: Chemical,* 51, 100-106.

Marquette, C. A. & Blum, L. J. 2000. Regenerable immunobiosensor for the chemiluminescent flow injection analysis of the herbicide 2,4-D. *Talanta,* 51, 395-401.

Martinez, R. C., Gonzalo, E. R., Santiago Toribio, M. P. & Mendez, J. H. 1996. Determination of triazines in surface waters by membrane separation coupled by on-line to a flow-injection system partial least square regression. *Analytica Chimica Acta,* 321, 147-155.

Mauriz, E., Calle, A., Abad, A., Montoya, A., Hildebrandt, A., Barcel, D. & Lechuga, L. M. 2006. Determination of carbaryl in natural water samples by a surface plasmon resonance flow-through immunosensor. Biosensors and Bioelectronics, 21, 2129-2136.

Mauriz, E., Calle, A., Manclis, J. J., Montoya, A., Escuela, A. M., Sendra, J. R. & Lechuga, L. M. 2006. Single and multi-analyte surface plasmon resonance assays for simultaneous detection of cholinesterase inhibiting pesticides. Sensors and Actuators B: Chemical, 118, 399-407.

Mauriz, E., Calle, A., Montoya, A. & Lechuga, L. M. 2006. Determination of environmental organic pollutants with a portable optical immunosensor. Talanta, 69, 359-364.

Mclaughlin, R. A., Michael Barringer, V., Brady, J. F., Yokley, R. A., Homer, M. L., Janis, E. M. & Orvin, C. B. 2008. Methods of Analysis for Triazine Herbicides and Their Metabolites. *The Triazine Herbicides.* San Diego: Elsevier.

Mellanby, K. 1992. The DDT Story. British Crop Protection Council.

Melquiades, F. L., Parreira, P. S., Yabe, M. J., Corazza, M. Z., Funfas, R. & Appoloni, C. R. 2007. Factorial design for Fe, Cu, Zn, Se and Pb preconcentration optimization with APDC and analysis with a portable X-ray fluorescence system. *Talanta,* 73, 121-126.

Mendez, J. H., Martinez, R. C., Dominguez, F. B. & Jimenez, J. I. J. 1988. Determination of the pesticides fenthion and fenitrothion by flow injection with amperometric detection. *Analytica Chimica Acta,* 209, 205-212.

Mezcua, M., Agüera, A., Lliberia, J. L., Cortés, M. A., Bagó, B. & Fernández-Alba, A. R. 2006. Application of ultra performance liquid chromatography-tandem mass spectrometry to the analysis of priority pesticides in groundwater. *Journal of Chromatography A,* 1109, 222-227.

Minitab. 2008. Minitab User Manual. Minitab, Inc., USA.

Mitchell, C., Brodie, J. & White, I. 2005. Sediments, nutrients and pesticide residues in event flow conditions in streams of the Mackay Whitsunday Region, Australia. *Marine Pollution Bulletin,* 51, 23-36.

Moore, A. & Lower, N. 2001. The impact of two pesticides on olfactory-mediated endocrine function in mature male Atlantic salmon (Salmo salar L.) parr. *Comparative Biochemistry and Physiology, Part B,* 129, 269-276.

Morris, T. 2008a. *Atrazine contamination of Hobarts drinking water* [Online]. The Greens political party. Available: http://www.tas.greens.org.au/News/view_MR.php?ActionID=3451 [Accessed 10/1 2009].

Morris, T. 2008b. *New report reveals atrazine poisons humans* [Online]. The Greens political party. Available: http://www.tas.greens.org.au/News/view_MR.php?ActionID=3002 [Accessed 10/1 2009].

Mostofa, K. M. G., Yoshioka, T., Konohira, E. & Tanoue, E. 2007. Dynamics and characteristics of fluorescent dissolved organic matter in the groundwater, river and lake water. *Water Air and Soil Pollution,* 184, 157-176.

Mostofa, K. M. G., Yoshioka, T., Konohira, E., Tanoue, E., Hayakawa, K. & Takahashi, M. 2005. Three-dimensional fluorescence as a tool for investigating the dynamics of dissolved organic matter in the Lake Biwa watershed. *Limnology,* 6, 101-115.

Mouvet, C., Amalric, L., Broussard, S., Lang, G., Brecht, A. & Gauglitz, G. 1996. Reflectometric Interference Spectroscopy for the Determination of Atrazine in Natural Water Samples. *Environmental Science & Technology,* 30, 1846-1851.

Mouvet, C., Harris, R. D., Maciag, C., Luff, B. J., Wilkinson, J. S., Piehler, J., Brecht, A., Gauglitz, G., Abuknesha, R. & Ismail, G. 1997. Determination of simazine in water samples by waveguide surface plasmon resonance. *Analytica Chimica Acta,* 338, 109-117.

Muller, J. F., Duquesne, S., Ng, J., Shaw, G. R., Krrishnamohan, K., Manonmanii, K., Hodge, M. & Eaglesham, G. K. 2000. Pesticides in sediments from Queensland irrigation channels and drains. *Marine Pollution Bulletin,* 41, 294-301.

Nadzialek, S., Spanò, L., Mandiki, S. N. M. & Kestemont, P. 2008. High doses of atrazine do not disrupt activity and expression of aromatase in female gonads of juvenile goldfish (Carassius auratus L.). *Ecotoxicology,* 17, 464-470.

Nagy, G., Fehér, Z. & Pungor, E. 1970. Application of silicone rubber-based graphite electrodes for continuous flow measurements : Part II. Voltammetric study of active substances injected into electrolyte streams. *Analytica Chimica Acta,* 52, 47-54.

NEMI. 2008. *National Environmental Methods index* [Online]. Available: http://www.nemi.gov/apex/ [Accessed 10/12 2008].

Neufeld, T., Eshkenazi, I., Cohen, E. & Rishpon, J. 2000. A micro flow injection electrochemical biosensor for organophosphorus pesticides. *Biosensors and Bioelectronics,* 15, 323-329.

NHMRC–NRMMC (National Health and Medical Research Council and Natural Resource Management Ministerial Council). 2004. Australian Drinking Water Guidelines, NHMRC and NRMMC, Canberrra.

NHMRC–NRMMC 2004. Australian Drinking Water Guidelines. *In:* Government, A. (ed.).

Nieves-Puigdoller, K., Björnsson, B. T. & Mccormick, S. D. 2007. Effects of hexazinone and atrazine on the physiology and endocrinology of smolt development in Atlantic salmon. *Aquatic Toxicology,* 84, 27-37.

Noppe, H., Ghekiere, A., Verslycke, T., Wulf, E. D., Verheyden, K., Monteyne, E., Polfliet, K., Caeter, P., Janssen, C. R. & De Brabander, H. F. 2007. Distribution and ecotoxicity of chlorotriazines in the Scheldt Estuary (B-Nl). *Environmental Pollution,* 147, 668-676.

O'connor 2005. Melbourne Water - Mid Yarra Catchment Water Quality Risk Assessment. Victoria: Ecos Environmental Consulting Pty Ltd.

O'connor 2008. Barwon Water - Moorabool Catchment Water Quality Risk Assessment. Victoria: Ecos Environmental Consulting Pty Ltd.

Ochsenbein, U., Zeh, M. & Berset, J.-D. 2008. Comparing solid phase extraction and direct injection for the analysis of ultra-trace levels of relevant explosives in lake water and tributaries using liquid chromatography-electrospray tandem mass spectrometry. *Chemosphere,* 72, 974-980.

Oliver, D. P., Kookana, R. S. & Salama, R. B. 2003a. Land use effects on sorption of pesticides and their metabolites in sandy soils. I. Fenamiphos and two metabolites, fenamiphos sulfoxide and fenamiphos sulfone, and fenarimol and azinphos methyl. *Australian Journal of Soil Research,* 41, 847-860.

Oliver, D. P., Kookana, R. S., Salama, R. B. & Correll, R. 2003b. Land use effects on sorption of pesticides and their metabolites in sandy soils. II. Atrazine and two metabolites, deethylatrazine and deisopropylatrazine, and prometryne. *Australian Journal of Soil Research,* 41, 861-874.

Önnerfjord, P., Eremin, S., Emnéus, J. & Marko-Varga, G. 1998. Fluorescence polarisation for immunoreagent characterisation. *Journal of Immunological Methods,* 213, 31-39.

Oroszlan, P., Duveneck, G. L., Ehrat, M. & Widmer, H. M. 1993. Fiber-optic Atrazine immunosensor. *Sensors and Actuators B: Chemical,* 11, 301-305.

Ottinger, M. A., Lavoie, E., Thompson, N., Barton, A., Whitehouse, K., Barton, M., Abdelnabi, M., Quinn, M., Panzica, G. & Viglietti-Panzica, C. 2008. Neuroendocrine and behavioral effects of embryonic exposure to endocrine disrupting chemicals in birds. *Brain Research Reviews,* 57, 376-385.

Palma, G., Sanchez, A., Olave, Y., Encina, F., Palma, R. & Barra, R. 2004. Pesticide levels in surface waters in an agricultural-forestry basin in Southern Chile. *Chemosphere,* 57, 763-770.

Parlanti, E., Worz, K., Geoffroy, L. & Lamotte, M. 2000. Dissolved organic matter fluorescence spectroscopy as a tool to estimate biological activity in a coastal zone submitted to anthropogenic inputs. *Organic Geochemistry,* 31, 1765-1781.

Perez-Ruiz, T., Lozano, C. M., Tomas, V. & Martin, J. 2002. Chemiluminescence determination of carbofuran and promecarb by flow injection analysis using two photochemical reaction. *Analyst,* 127, 1526.

Perez-Ruiz, T., Lozano, C. M., Tomas, V. & Martin, J. 2003. Flow injection chemiluminescence determination of carbaryl using photolytic decomposition and photogenerated tris (2,2′-bipyridyl)ruthenium(III). *Analytica Chimica Acta,* 476, 141-148.

Pérez-Ruiz, T., Martínez-Lozano, C. & García-Martínez, M. D. 2008. Simultaneous determination of amiodarone and its metabolite desethylamiodarone by high-performance liquid chromatography with chemiluminescent detection. *Analytica Chimica Acta,* 623, 89-95.

Pérez-Ruiz, T., Martínez-Lozano, C. & García, M. D. 2007a. Determination of N-methylcarbamate pesticides in environmental samples by an automated solid-phase extraction and liquid chromatographic method based on post-column photolysis and chemiluminescence detection. *Journal of Chromatography A,* 1164, 174-180.

Pérez-Ruiz, T., Martínez-Lozano, C. & García, M. D. 2007b. High-performance liquid chromatography-post-column chemiluminescence determination of aminopolycarboxylic acids at low concentration levels using tris(2,2'-bipyridyl)ruthenium(III). *Journal of Chromatography A,* 1169, 151-157.

Pérez-Ruiz, T., Martínez-Lozano, C., Tomás, V. & Fenoll, J. 2003. Chemiluminescence determination of citrate and pyruvate and their mixtures by the stopped-flow mixing technique. *Analytica Chimica Acta,* 485, 63-72.

Pérez-Ruiz, T., Martínez-Lozano, C., Tomás, V. & Martín, J. 2005. Automated solid-phase extraction and high-performance liquid chromatographic determination of nitrosamines using post-column photolysis and tris(2,2'-bipyridyl) ruthenium(III) chemiluminescence. *Journal of Chromatography A,* 1077, 49-56.

Pérez-Ruiz, T. S., Martinez-Lozano, C., Tom·S, V. & Casaj˙S, R. 1996. Flow-injection fluorimetric determination of nabam and metham. *Talanta,* 43, 193-198.

Perkins, B. L., Bushway, R. J. & Katz, L. E. 1999. Determination of hexazinone in ground water by direct injection high-performance liguid chromatography. *Journal of AOAC,* 82.

Peterson, D., Regehr, D. & Thompson, C. 2001. Herbicide Mode of Action. *In:* Extension, K. S. U. R. (ed.). Kansas State University.

Piehler, J., Brecht, A., Giersch, T., Hock, B. & Gauglitz, G. 1997. Assessment of affinity constants by rapid solid phase detection of equilibrium binding in a flow system. *Journal of Immunological Methods,* 201, 189-206.

Pogacnik, L. & Franko, M. 1999. Determination of organophosphate and carbamate pesticides in spiked samples of tap water and fruit juices by a biosensor with photothermal detection. *Biosensors and Bioelectronics,* 14, 569-578.

Pretty, J., Glaser, R., Jones, J. & Lunsford, R. A. 2004. A technique for the identification and direct analysis of hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine in metalworking fluids using electrospray-mass spectrometry. *Analyst,* 129, 1150-1155.

Pulgarin, J. A. M., Molina, A. A. & Lopez, P. F. 2006. Automatic chemiluminescence-based determination of carbaryl in various types of matrices. *Talanta,* 68(3):586-93.

Pullin, M. J., Anthony, C. & Maurice, P. A. 2007. Effects of iron on the molecular weight distribution, light absorption, and fluorescence properties of natural organic matter. *Environmental Engineering Science,* 24, 987-997.

Pyett, A. 2011. Floods threaten Australia's Great Barrier Reef. *Nature News* [Online]. Available: http://www.reuters.com/article/2011/01/10/us-australia-floods-reef-idUSTRE7091MH20110110 [Accessed 1/2].

Quintás, G., Garrigues, S. & De La Guardia, M. 2004. FT-Raman spectrometry determination of Malathion in pesticide formulations. *Talanta,* 63, 345-350.

Radcliff, G. W. 2002. *Pesticide use in Australia,* Chichester, J Wiley and sons.

Rao, Z., Zhang, X. & Baeyens, W. R. G. 2002. Chemiluminescence flow injection analysis of 1,3-dichloro-5,5-dimethylhydantoin in swimming pool water. *Talanta,* 57, 993-998.

Raven, P. & Johnson, G. 1992. *Biology*, Mosby Year Book.

Ridlen, J. S., Klopf, G. J. & Nieman, T. A. 1997. Determination of glyphosate and related compounds using HPLC with tris(2,2'-bipyridyl)ruthenium(II) electrogenerated chemiluminescence detection. *Analytica Chimica Acta,* 341, 195-204.

Rippeth, J. J., Gibson, T. D., Hart, J. P., Hartley, I. C. & Nelson, G. 1997. Flow-injection detector incorporating a screen-printed disposable amperometric biosensor for monitoring organophosphate pesticides. *Analyst,* 122, 1425-1430.

Rodriguez Jr, M., Sanders, C. A. & Greenbaum, E. 2002. Biosensors for rapid monitoring of primary-source drinking water using naturally occurring photosynthesis. *Biosensors and Bioelectronics,* 17, 843-849.

Ruedas Rama, M. J., Ruiz Medina, A. & Molina Díaz, A. 2004. Bead injection spectroscopy-flow injection analysis (BIS-FIA): an interesting tool applicable to pharmaceutical analysis: Determination of promethazine and trifluoperazine. *Journal of Pharmaceutical and Biomedical Analysis,* 35, 1027-1034.

*Tasmania: Name your Poison,* Year. Television. Directed by Rushworth, N. Australia: Nine Network.

Ruzicka, J. & Hansen, E. H. 1975. Flow injection analyses : Part I. A new concept of fast continuous flow analysis. *Analytica Chimica Acta,* 78, 145-157.

Ruzicka, J. & Hansen, E. H. 1988. *Flow Injection Analysis*, Wiley & Sons.

*Flow: for Love of Water,* Year. DVD. Directed by Salina, I. USA.

Salinas-Castillo, A., Fernández-Sánchez, J. F., Segura-Carretero, A. & Fernández-Gutiérrez, A. 2004. A facile flow-through phosphorimetric sensing device for simultaneous determination of naptalam and its metabolite 1-naphthylamine. *Analytica Chimica Acta,* 522, 19-24.

Santin, C., Yamashita, Y., Otero, X. L., Alvarez, M. A. & Jaffe, R. 2009. Characterizing humic substances from estuarine soils and sediments by excitation-emission matrix spectroscopy and parallel factor analysis. *Biogeochemistry,* 96, 131-147.

Satínský, D., Huclová, J., Ferreira, R. L. C., Montenegro, M. C. B. S. M. & Solich, P. 2006. Determination of ambroxol hydrochloride, methylparaben and benzoic acid in pharmaceutical preparations based on sequential injection technique coupled with monolithic column. *Journal of Pharmaceutical and Biomedical Analysis,* 40, 287-293.

Satínský, D., Santos, L. M. L. D., Sklenárová, H., Solich, P., Montenegro, M. C. B. S. M. & Araújo, A. N. 2005. Sequential injection chromatographic determination of ambroxol hydrochloride and doxycycline in pharmaceutical preparations. *Talanta,* 68, 214-218.

Satínský, D., Solich, P., Chocholous, P. & Karlícek, R. 2003. Monolithic columns--a new concept of separation in the sequential injection technique. *Analytica Chimica Acta,* 499, 205-214.

Saurina, J. 2008. *Multicomponent Flow Injection Analysis*, Wiley-VCH Verlag GmbH & Co. KGaA.

Schobel, U., Barzen, C. & Gauglitz, G. 2000. Immunoanalytical techniques for pesticide monitoring based on fluorescence detection. *Fresenius' Journal of Analytical Chemistry,* 366, 646-658.

Schoning, M. J., Krause, R., Block, K., Musahmeh, M., Mulchandani, A. & Wang, J. 2003. A dual amperometric/potentiometric FIA-based biosensor for the distinctive detection of organophosphorus pesticides. *Sens. Actuat. B,* 95, 291-296.

Sendra, B., Panadero, S., Eremin, S. & Gómez-Hens, A. 1998. Kinetic determination of atrazine in foods based on stopped-flow fluorescence polarization immunoassay. *Talanta,* 47, 153-160.

Shaw, M., Furnas, M. J., Fabricius, K., Haynes, D., Carter, S., Eaglesham, G. & Mueller, J. F. 2010. Monitoring pesticides in the Great Barrier Reef. *Marine Pollution Bulletin,* 60, 113-122.

Shaw, M. & Muller, J. F. 2005. Preliminary evaluation of the occurrence of herbicides and PAHs in the Wet Tropics region of the Great Barrier Reef, Australia, using passive samplers. *Marine Pollution Bulletin,* 51, 876-881.

Shaw, M., Negri, A., Fabricius, K. & Mueller, J. F. 2009. Predicting water toxicity: Pairing passive sampling with bioassays on the Great Barrier Reef. *Aquatic Toxicology,* 95, 108-116.

Shi, R. & Stein, K. 1996. Flow injection analysis of paraoxon with the use of an immobilized acetylcholinesterase reactor. *Analytica Chimica Acta,* 324, 21-27.

Shitanda, I., Takamatsu, S., Watanabe, K. & Itagaki, M. 2009. Amperometric screen-printed algal biosensor with flow injection analysis system for detection of environmental toxic compounds. Electrochimica Acta, 54, 4933-4936.

Sih, A., Bell, A. M. & Kerby, J. L. 2004. Two stressors are far deadlier than one. *Trends in Ecology & Evolution,* 19, 274-276.

Solarska, S., May, T., Roddick, F. A. & Lawrie, A. C. 2009. Isolation and screening of natural organic matter-degrading fungi. *Chemosphere,* 75, 751-758.

Solich, P., Spas, D. K. & Ian, D. M. 2008. Chapter 10 Chromatographic Separations. *Comprehensive Analytical Chemistry.* Elsevier.

Solná, R., Sapelnikova, S., Skládal, P., Winther-Nielsen, M., Carlsson, C., Emnéus, J. & Ruzgas, T. 2005. Multienzyme electrochemical array sensor for determination of phenols and pesticides. *Talanta,* 65, 349-357.

Spark, K. M. & Swift, R. S. 1994. Investigation of the interaction between pesticides and humic substances using fluoresence spectroscopy. *Science of the Total Environment,* 152, 9-17.

Srinivasan, P. 2003. Paraquat: A unique contributor to agriculture and sustainable development. New Deli: Indian Institute of Management.

Starodub, N. F., Kanjuk, N. I., Kukla, A. L. & Shirshov, Y. M. 1999. Multi-enzymatic electrochemical sensor: field measurements and their optimisation. *Analytica Chimica Acta,* 385, 461-466.

Stoker, T. E., Kavlock, R. J. & Robert, K. 2010. Pesticides as Endocrine-Disrupting Chemicals. *Hayes' Handbook of Pesticide Toxicology (Third Edition).* New York: Academic Press.

Storrs, S. I. & Kiesecker, J. M. 2004b. Survivorship Patterns of Larval Amphibians Exposed to Low Concentrations of Atrazine *Environmental Health Perspectives,* 112, 1054-1057.

Stutchbury, B. 2008. Did your shopping list kill a Song Bird? *The New York Times*, 30/3/2008.

Suwansa-Ard, S., Kanatharana, P., Asawatreratanakul, P., Limsakul, C., Wongkittisuksa, B. & Thavarungkul, P. 2005. Semi disposable reactor biosensors for detecting carbamate pesticides in water. *Biosensors and Bioelectronics,* 21, 445-454.

Tanaka, T., Kohno, H., Suzuki, R. & Sugie, S. 2004. Lack of modifying effects of an estrogenic compound atrazine on 7,12-dimethylbenz(a)anthracene-induced ovarian carcinogenesis in rats. *Cancer Letters,* 210, 129-137.

Tariq, M. I., Afzal, S. & Hussain, I. 2004. Pesticides in shallow groundwater of Bahawalnagar, Muzafargarh, D.G. Khan and Rajan Pur districts of Punjab, Pakistan. *Environment International,* 30, 471-479.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fournier, M., Cyr, D. & Marcogliese, D. 2002. Response of the amphibian tadpole (Xenopus Laevis) to atrazine during sexual differentiation of the testis. *Environmental Toxicology and Chemistry,* 21, 527-531.

Taylor, G. 1953. Dispersion of Soluble Matter in Solvent Flowing Slowly through a Tube. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences (1934-1990),* 219, 186-203.

Taylor, G. 1954. The Dispersion of Matter in Turbulent Flow through a Pipe. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences (1934-1990),* 223, 446-468.

Tebbutt, P. 1998. *Basic Mathematics for Chemists*, Wiley.

Thomas, A. 2011. Reefs reelin from Queensland floods. Available: http://www.abc.net.au/news/stories/2011/01/21/3118234.htm [Accessed 1/2].

Townshend, A., Ruengsitagoon, W., Thongpoon, C. & Liawruangrath, S. 2004. Flow Injection Chemiluminescence determination of teracycline. *Analytica Chimica Acta,* 541, 103-109.

Trajkovska, V., Petrovska-Jovanovic, S. & Cvetkovski, M. 2001. Development and optimization of a method for the determination of simazine, atrazine and propazine using solid-phase extraction and HPLC/GC. *Journal of the Serbian Chemical Society,* 66, 199-204.

Tran, A. T. K., Hyne, R. V. & Doble, P. 2007. Determination of commonly used polar herbicides in agricultural drainage waters in Australia by HPLC. *Chemosphere,* 67, 944-953.

Trojanowicz, M. 2000. *Flow Injection Analysis: Instrumentation and Applications*, World Scientific Publishing.

Tsogas, G. Z., Giokas, D. L., Nikolakopoulos, P. G., Vlessidis, A. G. & Evmiridis, N. P. 2009. Determination of the pesticide carbaryl and its photodegradation kinetics in natural waters by flow injection-direct chemiluminescence detection. *Analytica Chimica Acta,* 573-574, 354-359.

Tudorache, M., Tencaliec, A. & Bala, C. 2008. Magnetic beads-based immunoassay as a sensitive alternative for atrazine analysis. *Talanta,* 77, 839-843.

Turiel, E., Fernández, P., Pérez-Conde, C., Gutiérrez, A. M. & Cámara, C. 1999. Oriented antibody immobilization for atrazine determination by a flow-through fluoroimmunosensor. *Fresenius' Journal of Analytical Chemistry,* 365, 658-662.

Turiel, E., Fernández, P., Pérez-Conde, C., Gutiérrez, A. M. & Cámara, C. 1998. Flow-through fluorescence immunosensor for atrazine determination. Talanta, 47, 1255-1261.

Ueoka, M., Allinson, G., Kelsall, Y., Graymore, M. & Stagnitti, F. 1997. Environmental fate of pesticides used in Australian viticulture: Behaviour of dithianon and vinclozolin in the soils of the South Australian Riverland. *Chemosphere,* 35, 2915-2924.

USEPA. 1999. *United States Environmental Protection Agency: Dicrotophos guidelines* [Online]. Available: http://www.epa.gov/pesticides/op/dicrotophos/hed-dicro.pdf [Accessed 1/1 2005].

USEPA. 2008a. *Analytical Methods developed by the office of Ground Water and Drinking Waetr* [Online]. Available: http://www.epa.gov/safewater/methods/analyticalmethods_ogwdw.html [Accessed 2008].

USEPA. 2008b. *History of the US EPA: 1970s* [Online]. EPA. [Accessed 1/1/2009].

Valcárcel, M. & Luque De Castro, M. D. 1987. *Flow Injection Analysis: Principles and Applications,* Spain, Ellis Horwood Limited. Wiley & Sons.

Valdes-Ramirez, G., Gutirrez, M., del Valle, M., Ramirez-Silva, M. T., Fournier, D. & Marty, J. L. 2009. Automated resolution of dichlorvos and methylparaoxon pesticide mixtures employing a Flow Injection system with an inhibition electronic tongue. Biosensors and Bioelectronics, 24, 1103-1108.

Varsamis, D. G., Touloupakis, E., Morlacchi, P., Ghanotakis, D. F., Giardi, M. T. & Cullen, D. C. 2008. Development of a photosystem II-based optical microfluidic sensor for herbicide detection. Talanta, 77, 42-47.

Vilchez, J. L., Valencia, M. C., Navalon, A., Molinero-Morales, B. & Capitan-Vallvey, L. F. 2001. Flow injection analysis of the insecticide imidacloprid in water samples with photochemically induced fluorescence detection. *Analytica Chimica Acta,* 439, 299-305.

Wang, J., Zhang, C., Wang, H., Yang, F. & Zhang, X. 2001. Development of a luminol-based chemiluminescence flow-injection method for the determination of dichlorvos pesticide. *Talanta,* 54, 1185-1193.

Wang, L. Y., Wu, F. C., Zhang, R. Y., Li, W. & Liao, H. Q. 2009a. Characterization of dissolved organic matter fractions from Lake Hongfeng, Southwestern China Plateau. *Journal of Environmental Sciences-China,* 21, 581-588.

Wang, X., Lin, J.-M., Liu, M.-L. & Cheng, X.-L. 2009b. Flow-based luminescence-sensing methods for environmental water analysis. *TrAC Trends in Analytical Chemistry,* 28, 75-87.

Ware, G. 2000. *The Pesticide Book*, Sephard Poorman Inc.

West, W., Muller, R. H. & Jette, E. 1928. Studies on Fluorescence and Photosensitization in Aqueous Solution. I. Introduction. *Proceedings of the Royal Society of London. Series A, Containing Papers of a Mathematical and Physical Character,* 121, 294-298.

Wilhelms, K. W., Cutler, S. A., Proudman, J. A., Anderson, L. L. & Scanes, C. G. 2005. Atrazine and the Hypothalamo-Pituitary-Gonadal Axis in Sexually Maturing Precocial Birds: Studies in Male Japanese Quail. *Toxicol. Sci.,* 86, 152-160.

Wilhelms, K. W., Cutler, S. A., Proudman, J. A., Carsia, R. V., Anderson, L. L. & Scanes, C. G. 2006. Lack of effects of atrazine on estrogen-responsive organs and circulating hormone concentrations in sexually immature female Japanese quail (Coturnix coturnix japonica). *Chemosphere,* 65, 674-681.

Wilson, R., Barker, M. H., Schiffrin, D. J. & Abuknesha, R. 1997. Electrochemiluminescence flow injection immunoassay for atrazine. *Biosensors and Bioelectronics,* 12, 277-286.

Wittmann, C. 1996. Immunochemical Techniques and Immunosensors for the Analysis of Dealkylated Degradation Products of Atrazine. *International Journal of Environmental Analytical Chemistry,* 65, 113-126.

Wittmann, C. & Schmid, R. D. 1994. Development and Application of an Automated Quasi-Continuous Immunoflow Injection System to the Analysis of Pesticide Residues in Water and Soil. *Journal of Agricultural and Food Chemistry,* 42, 1041-1047.

Worm, O. & Vaupel, K. 2009. Dirty portfolios of pesticide companies. Greenpeace (ed.).

Wortberg, M., Middendorf, C., Katerkamp, A., Rump, T., Krause, J. & Cammann, K. 1993. Flow-injection immunosensor for triazine herbacides using Eu(III) chelate label fluorescence detrection. *Analytica Chimica Acta,* 289, 177-186.

Wu, F. C., Midorikawa, T. & Tanoue, E. 2001. Fluorescence properties of organic ligands for copper(II) in Lake Biwa and its rivers. *Geochemical Journal,* 35, 333-346.

Xu, H., Duan, C., Zhang, Z., Chen, J., Lai, C., Lian, M., Liu, L. & Cui, H. 2004. Flow injection determination of p-aminophenol at trace level using inhibited luminol-dimethylsulfoxide-NaOH-EDTA chemiluminescence. *Water Research,* 39, 396-402.

Yakovleva, J., Davidsson, R., Bengtsson, M., Laurell, T. & Emnéus, J. 2003. Microfluidic enzyme immunosensors with immobilised protein A and G using chemiluminescence detection. *Biosensors and Bioelectronics,* 19, 21-34.

Yamashita, Y. & Jaffe, R. 2008. Characterizing the interactions between trace metals and dissolved organic matter using excitation-emission matrix and parallel factor analysis. *Environmental Science & Technology,* 42, 7374-7379.

Yamashita, Y. & Tanoue, E. 2003. Chemical characterization of protein-like fluorophores in DOM in relation to aromatic amino acids. *Marine Chemistry,* 82, 255-271.

Yamazaki, S., Shinozaki, T. & Tanimura, T. 1998. Detection of Tertiary Amines with Chemiluminescent Reaction Using Tris(2,2′-bipyridine)ruthenium(III) Prepared by On-Line Photochemical Oxidation. *Journal of High Resolution Chromatography,* 21, 315-316.

Yaqoob, M., Nabi, A. & Worsfold, P. J. 2004a. Determination of nanomolar concentrations of phosphate in freshwaters using flow injection with luminol chemiluminescence detection. *Analytica Chimica Acta,* 510, 213-218.

Yaqoob, M., Nabi, A. & Worsfold, P. J. 2004b. Determination of silicate in freshwaters using flow injection with luminol chemiluminescence detection. *Analytica Chimica Acta,* 519, 137-142.

Zambonin, C. G. & Palmisano, F. 2000. Determination of triazines in soil leachates by solid-phase microextraction coupled to gas chromatography-mass spectrometry. *Journal of Chromatography A,* 874, 247-255.

Zhou, Q. X., Pang, L., Xie, G. H., Xiao, J. P. & Bai, H. H. 2009. Determination of Atrazine and Simazine in Environmental Water Samples by Dispersive Liquid-Liquid Microextraction with High Performance Liquid Chromatography. *Analytical Sciences,* 25, 73-76.

Zhou, Q. X., Xie, G. H. & Pang, L. 2008. Rapid determination of atrazine in environmental water samples by a novel liquid phase microextraction. *Chinese Chemical Letters,* 19, 89-91.

Zhu, Y. & Li, Q. X. 2002. Movement of bromacil and hexazinone in soils of Hawaiian pineapple fields. *Chemosphere,* 49, 669-674.